

Bidirectional Interaction between Farnesoid X Receptor and MicroRNAs

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ABSTRACT

Little is known about the bidirectional interaction between the bile acid (BA) sensor farnesoid X receptor (FXR) and microRNAs (miRNAs) in diseases of the human digestive system. The nuclear receptor FXR has been shown to play a key role in the regulation of metabolic pathways, inflammatory response and cell cycle regulation in liver and intestine. MiRNAs are currently gaining interest as potential drug targets, because they regulate the expression of approximately 60% of all human genes and they appear to be at the center of the balance between cell proliferation and apoptosis. Thus, miRNA molecules are currently entering the clinical test phase as putative future treatment options for several diseases of the human digestive system such as steatohepatitis, cholestasis, (cholangio)hepatocellular carcinoma or colon cancer. The ability of miRNAs to simultaneously influence the expression of a large set of genes is controversially discussed in the literature. This “one-to-multiple” relationship of miRNAs can be beneficial in multipathway diseases such as cancer, where several oncogenes can be miRNA-dependently suppressed. In contrast, the risk for drug-associated undesired off-target effects is increased by targeting a miRNA as compared to influencing the target gene itself. Here we elucidate how miRNAs are involved in the regulation of FXR expression and influence the physiological and pathophysiological role of FXR. Based on the results, we further discuss miRNAs as potential drug targets and therapeutic agents for liver and digestive diseases.

This thesis consists of three parts. In the first part, we show that strongest activation of the BA-FXR axis significantly influences the expression of 81 miRNAs and 2304 genes, all of which are known to be involved in hepatic lipid, BA and drug metabolism. Further, the p53-regulated, apoptotic miRNA miR-34a is BA-dependently suppressed and shows an inverse expression correlation with several genes important for the maintenance of BA homeostasis. Based on this finding we suggest that miR-34a plays a key role in the autoregulation of BA homeostasis, conferring a link between BA dysregulation and apoptosis in dependence of FXR transactivation. In the second part, we demonstrate that FXR itself is epigenetically regulated by miR-192 in hepatoma and colon cancer-derived cell lines. MiR-192 is, like miR-34a, a p53-regulated miRNA known to be important in cell proliferative and apoptotic processes. We further show that also miR-192 has the potential to influence BA homeostasis by FXR-dependent suppression of the expression of relevant BA transporters such as BSEP, MRP2 and OST α/β . Additionally, miR-192-3p transfection results in a partially FXR-

dependent, decreased proliferation of Huh-7 cells, whereas miR-192 also shows some FXR-independent effects on cell viability and cell cycle regulatory pathways. In the last part of this thesis, we focus on the miRNA-dependent regulation of the FXR-controlled drug transporter OATP1B3. We reveal that OATP1B3 expression is directly regulated by three miRNAs, miR-509-5p/-3p and miR-656, whereas miR-192-5p and -3p also suppress OATP1B3 expression in a FXR-dependent manner. By this “multi-to-one” relationship of miRNA-dependent regulation of OATP1B3 expression, the complexity of the endogenous miRNA-dependent gene regulatory network is discussed.

In summary, we demonstrate that a bidirectional interaction between the bile acid sensor FXR and miRNAs is of importance for physiological and pathological effects on the human digestive system. The complexity of the endogenous miRNA network may have important consequences for the development of miRNA therapeutics. With focus on the p53-regulated miRNAs miR-34a and miR-192, we discuss current problems in miRNA therapeutic development such as tissue specificity and undesired off-target effects. As the reproducibility of *in vitro* miRNA studies is low, early performance of human studies is inevitable. We conclude that miRNAs such as miR-192 or miR-34a are high-value drug targets and agents, when targeted drug delivery to the diseased tissue can be achieved. A drug distribution of mimics into healthy tissue should be avoided for both p53-regulated miRNAs, as they have the potential to disturb the BA-FXR axis and may decrease cell viability. Since not all mechanisms of miRNA-dependent gene regulation are probably discovered yet, continuous miRNA research is still essential for the development of truly innovative and safe treatment concepts.

ZUSAMMENFASSUNG

Über die bidirektionale Interaktion zwischen dem Gallensäure-Sensor Farnesoid-X Rezeptor (FXR) und microRNAs (miRNAs) bei Erkrankungen des menschlichen Verdauungsapparates ist nur wenig bekannt. Der Kernrezeptor FXR spielt eine Schlüsselrolle in der metabolischen Regulation, der Entzündungsantwort sowie in der Zellzyklen-Kontrolle in Leber und Darm. MiRNAs gewinnen als therapeutische Ansatzpunkte immer mehr an Bedeutung, da sie bis zu 60% der menschlichen Gene zu regulieren scheinen und eine zentrale Rolle im Gleichgewicht zwischen Zellwachstum und Apoptose einnehmen. Einzelne miRNA-Moleküle werden in klinischen Studien schon als therapeutische Arzneimittel gegen Erkrankungen wie Steatohepatitis, Cholestase, (cholangio)-hepatozelluläres Karzinom oder auch Kolonkarzinom getestet. Die Fähigkeit von miRNAs, zeitgleich die Expression von mehreren Genen regulieren zu können, wird in der Fachliteratur kontrovers diskutiert. Diese Fähigkeit kann zum Beispiel bei Krebserkrankungen, bei welchen meist mehrere Onkogene und entsprechende Signalwege dysreguliert sind, von Vorteil sein. Zum anderen besteht bei miRNA-basierten Therapeutika immer das unerwünschte Risiko für „off-target“ Effekte – wahrscheinlich mit einem höheren Risiko, als wenn man das Zielprotein selbst medikamentös beeinflussen würde. In dieser Dissertation wird veranschaulicht, wie miRNAs in der Expressionsregulierung von FXR und an dessen physiologischer und pathophysiologischer Wirkung beteiligt sind. Weiter wird auf Vor- und Nachteile von möglichen miRNA-Therapeutika bei Leber- und Darmerkrankungen eingegangen.

Diese schriftliche Arbeit besteht aus drei Teilen. Im ersten Kapitel wird veranschaulicht, dass die Expression von 81 miRNAs und 2304 Genen, welche im hepatischen Lipid-, Gallensäuren- und Medikamentenmetabolismus involviert sind, durch Aktivierung der Gallensäure/FXR-Achse signifikant beeinflusst wird. Die p53-regulierte, apoptotische miRNA miR-34a wird Gallensäure-abhängig supprimiert und zeigt eine inverse Expressionskorrelation mit mehreren Genen, welche für die Aufrechterhaltung der endogenen Gallensäurehomöostase wichtig sind. Anhand dieser Resultate können wir schlussfolgern, dass miR-34a eine potentielle Schlüsselrolle in der Autoregulation der Gallensäurehomöostase spielt. Weiter zeigt uns dieses Beispiel einen Zusammenhang zwischen Gallensäuredysregulation und Apoptosis in Abhängigkeit der Transaktivierung von FXR. Im zweiten Teil zeigen wir in Hepatom- und Kolonkarzinom-Zelllinien, dass die Expression von FXR durch miR-192 epigenetisch kontrolliert wird. MiR-192 gehört wie miR-34a zu den p53-gesteuerten miRNAs, welche für ihre Wichtigkeit in

zellproliferativen und apoptotischen Prozessen bekannt sind. Wir heben zudem hervor, dass auch miR-192 die Gallensäurehomöostase durch eine FXR-abhängige Suppression der Expression von relevanten Gallensäuretransportern (u.a. BSEP, MRP2 und OST α/β) potentiell negativ beeinflussen kann. Die Transfektion von miR-192-3p resultiert weiter in einem verminderten Wachstum von Huh-7 Zellen, wobei dieser miR-192-Effekt zu einem gewissen Anteil als FXR-abhängig interpretiert werden kann. MiR-192 zeigt jedoch auch FXR-unabhängige Effekte, z.B. auf die Zelllebensfähigkeit sowie auf Zellzyklus-regulatorische Signalwege von Huh-7 Zellen. Der letzte Teil dieser Dissertation fokussiert auf die miRNA-abhängige Regulation des FXR-kontrollierten Medikamententransporters OATP1B3. Wir zeigen, dass die Expression von OATP1B3 durch drei miRNAs direkt reguliert wird (miR-509-5p/-3p und miR-656) sowie, dass miR-192-5p und -3p indirekt, durch ihren Einfluss auf FXR, die OATP1B3 Expression herunterregulieren. Anhand dieses Beispiels, welches aufzeigt, dass die Expression von einem Gen von mehreren miRNAs gleichzeitig reguliert werden kann, diskutieren wir die Komplexität des endogenen miRNA-Netzwerks.

Zusammenfassend können wir mit dieser Arbeit hervorheben, dass eine bidirektionale Interaktion zwischen dem Gallensäure-Sensor FXR und miRNAs eine entscheidende Rolle in der Physiologie und Pathophysiologie des humanen Verdauungssystems einnimmt. Die veranschaulichte Komplexität des endogenen miRNA-Netzwerks könnte klinisch relevante Probleme während der Entwicklung von miRNA-Therapeutika hervorrufen. Mit Bezug auf die p53-regulierten miRNAs miR-34a und miR-192 veranschaulichen wir aktuelle Probleme in der miRNA-basierten Medikamentenentwicklung wie z.B. Zielstrukturspezifität sowie unerwünschte „off-target“ Effekte. Die Reproduzierbarkeit von miRNA-assoziierten *in vitro* Studien ist grundsätzlich sehr niedrig, womit eine zeitnahe Durchführung von Humanstudien unumgänglich ist. Wir können somit schlussfolgern, dass miRNAs wie miR-192 oder miR-34a hochwertige therapeutische Ansatzpunkte sind, falls eine zielgerichtete Medikamentenverteilung in erkranktes Gewebe möglich ist. Eine Verteilung in gesundes Gewebe sollte im Falle von p53-regulierten miRNA-Molekülen sicherlich vermieden werden, da die Gallensäure/FXR-Achse potentiell dysreguliert sowie die Lebensfähigkeit von gesunden Zellen vermindert werden kann. Da bis heute wahrscheinlich noch nicht alle Mechanismen der miRNA-abhängigen Genregulation bekannt sind, ist eine weiterführende miRNA Forschung für die zukünftige Entwicklung von innovativen und gut tolerierbaren miRNA-Therapeutika unabdingbar.

ABBREVIATIONS

ABC – ATP-binding cassette	DRIP – vitamin D-interacting protein
ACAT1 – acetyl-CoA acetyltransferase 1 gene	EtOH – ethanol
AGO – argonaute	FABP – fatty acid-binding protein
AGPAT2 – 1-acylglycerol-3-phosphate O-acetyltransferase 2	FASN – fatty acid synthase gene
AhR – arylhydrocarbon receptor	FDR – false discovery rate
AJCC – American Joint Committee on Cancer	FGF19 – fibroblast growth factor 19
AKR1C1 – aldo-keto reductase 1C1	FGFR4 – fibroblast growth factor receptor 4
APO – apolipoprotein	Foxm1b – forkheadbox m1b
ASBT – apical sodium dependent bile acid transporter	FOXO3 – forkheadbox O3
ATP – adenosine triphosphate	FXR – farnesoid X receptor
BA – bile acid	GIST – gastrointestinal stromal tumor
BCL-2 – B-cell lymphoma 2	GLM – generalized linear model
BSEP – bile salt export pump	GLP-1 – glucagon-like peptide 1
BSP – bromsulphthalein	HBV – hepatitis B virus
CA – cholic acid	HCC – hepatocellular carcinoma
CAR – constitutive androstane receptor	HCV – hepatitis C virus
CCA – cholangiocarcinoma	HDL – high density lipoprotein
CCK – cholecystokinin	HMGCS2 – 3-hydroxy-3-methylglutaryl-CoA synthase 2
CDCA – chenodeoxycholic acid	HNF – hepatocyte nuclear factor
CHC – hepatocellular-cholangiocarcinoma	HTCR – Human Tissue and Cell Research
CPT1A – carnitine palmitoyltransferase 1A	ICC – intrahepatic cholangiocarcinoma
CRC – colorectal cancer	IL – interleukin
CYP – cytochrome P450	JNK – c-jun n-terminal kinase
DCA – deoxycholic acid	LCA – lithocholic acid
DM – drug metabolism	LDL – low density lipoprotein
DME – drug metabolism enzymes	LDLR – low density lipoprotein receptor
DMSO – dimethyl sulfoxide	LPS – lipopolysaccharide
DNA – deoxyribonucleic acid	LXR – liver X receptor
	MDM2 – mouse double minute 2 homolog

MDR3 – multidrug resistance protein 3	RT-PCR – real-time polymerase chain reaction
miRNA/miR – microRNA	RXR – retinoid X receptor
mRNA – messenger RNA	S1PR – sphingosine-1-phosphate receptor
MRP2 – multidrug resistance-associated protein 2	SDS – sodium dodecyl sulfate
NAFLD – non-alcoholic fatty liver disease	SHP – small heterodimer partner
NASH – non-alcoholic steatohepatitis	SIP1 – smad interacting protein 1
NC – negative control	siRNA – small interfering RNA
NFκB – nuclear factor κ-light-chain-enhancer of activated B cells	SIRT1 – sirtuin-1
NGS – next generation sequencing	SLC – solute carrier family
NOS – nitric oxide synthase	SREBF2 – sterol regulatory element binding transcription factor 2
NPC1L1 – Niemann-Pick C1-like 1 gene	SREBP-1c – steroid response element binding protein 1c
NTCP – Na ⁺ -taurocholate cotransporting polypeptide.	STARD3 – StAR-related lipid transfer domain protein 3
OATP – organic anion transporting polypeptide	SULT – sulfotransferase
OCA – obeticholic acid	TCA – taurocholic acid
OST – organic solute transporter	TGF – transforming growth factor
PCR – polymerase chain reaction	TNF – tumor necrosis factor
PCSK9 – proprotein convertase subtilisin/kexin type 9	TSS – transcription start site
PFIC – progressive familial intrahepatic cholestasis	UDCA – ursodeoxycholic acid
PGC – peroxisome proliferator-activated receptor gamma coactivator	UGT – UDP-glucuronosyltransferase
PHH – primary human hepatocytes	UTR – untranslated region
PPAR – peroxisome proliferator-activated receptor	VEGFA – vascular endothelial growth factor A
PRKCA – protein kinase C alpha	VLDL – very low density lipoprotein
PXR – pregnane X receptor	ZEB2 – zinc finger E-box binding homeobox 2
RAR – retinoic acid receptor	
RISC – RNA-induced silencing complex	
RNA – ribonucleic acid	
ROS – reactive oxygen species	

I. INTRODUCTION

1. Physiology of the human digestive system

The human digestive system consists of the gastrointestinal tract, the liver, the pancreas, and the gallbladder. The digestive system helps the body to break down food into nutrients (carbohydrates, proteins, fat, and vitamins), which are used for energy, growth, and cell repair. Digestion requires the interplay between organs, hormones, and nerves involving the whole human body as such. The hormone cholecystokinin (CCK) for example, is a key regulator of food ingestion and digestion. During meal intake, it is secreted from the small intestine, where it facilitates the secretion of pancreatic enzymes and bile from the gallbladder [1]. Bile acids (BA), one of the main constituents of the bile, are potent digestive surfactants that promote the absorption of lipids and fat-soluble vitamins from the intestine into the circulatory system.

Orally taken drugs can enter the human body over the digestive system. For most of them, their bioavailability is dependent on transporters or metabolizing enzymes located in the liver or intestine. Many drugs are known to influence the activity of drug transporters or enzymes affecting the pharmacokinetics of other therapeutics, thus leading to drug-drug interactions. Some of these transporters and enzymes are as well important for the maintenance of BA homeostasis.

1.1 The liver

The liver is the largest organ of the human organism, constituting approximately 2-3% of average body weight. Being strongly vascularized, it receives up to 25% of the total cardiac output. The liver consists of four lobules and has a dual blood supply: the hepatic artery and the portal vein [2]. The classical structural unit of the liver is the hepatic lobule, having a shape of a polygon. The lobule is composed of the central vein in the center and the portal triad at the corners, consisting of the hepatic artery, the portal vein and the bile duct. Plates of hepatocytes radiate from the central vein to the perimeter of the lobule to define the hepatic acinus, the functional unit of the liver. The hepatic acinus is divided into three zones, categorizing the hepatocyte strains regarding their functional activity (Fig. 1) [3]. Nearly 80% of the total liver volume is constituted by hepatocytes. Other liver cells encompass cholangiocytes as epithelial cells of the intrahepatic bile ducts, liver sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells and pit cells such as intrahepatic lymphocytes or natural killer cells [4].

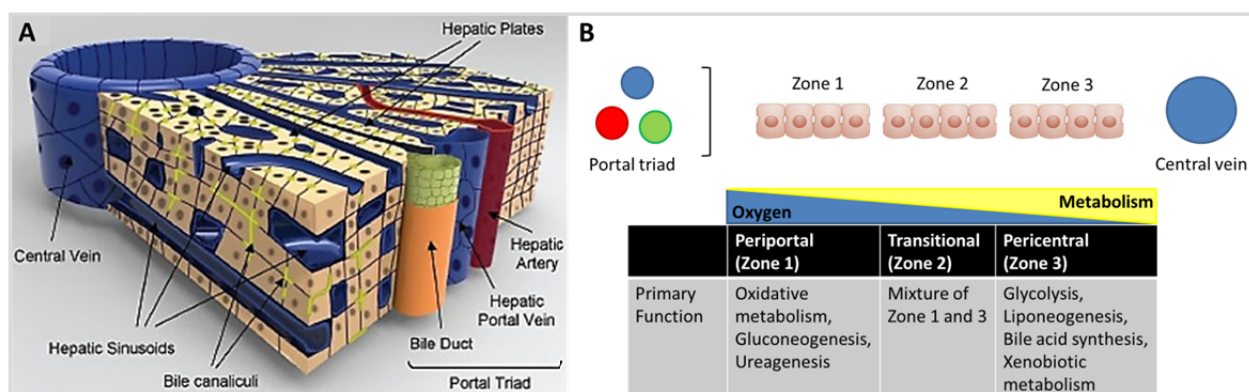


Figure 1: Three-dimensional architecture of the liver between the central vein and a portal triad (A), and functional zones of the hepatic acinus (B). The networks of bile canaliculi (yellow-green) run parallel and counter to the blood flow through the sinusoids. Adapted from [3].

The main liver functions are metabolic regulation, hematological regulation (e.g. production of coagulation proteins), and bile production. The liver is the most important detoxifying organ in human body, which metabolizes endogenous molecules and xenobiotics, and contributes to the clearance of drugs and toxins over the urine or feces. The liver is furthermore important for storage of nutrients such as glucose, fat-soluble vitamins or iron [5].

1.2 The intestine

The small intestine and colon (large intestine) are essential parts of the gut, whose main function is nutrient digestion and absorption as well as feces formation. The small intestine is structurally divided into duodenum, jejunum and ileum. To increase resorption ability, the small intestine contains crypts and villi leading to an intestinal surface enlargement. A tight capillary network, directly located under the epithelium, is important for a rapid resorption of nutrients and drugs [6]. The colon can be divided into four sections and its main task is the removal of water, salt and nutrients from the chyme for feces formation. Distinct subpopulations of intestinal epithelial cells are integrated into a continuous, single cell layer forming the epithelium. The epithelium is divided into apical and basolateral regions that are separated by tight junctions, forming a rigorous barrier between gut and blood system. Enterocytes form the main cells in the small intestine, and colonocytes the ones in the large intestine. Both cell types are polarized and show a typical apical brush border facing the intestinal lumen. The intestinal epithelium continuously produces mucus and is covered by it, which mainly serves as a defense against microbes. Mucus is generated by goblet cells, whereas mucus production is higher in colon as compared to small intestine. The thickness

and continuity of intestinal mucus differs regionally. It is thinner and discontinuous in the proximal small intestine and becomes thicker and continuous along the colon, showing some correlation with the local bacterial load (about 10^8 organisms/g in the ileum; about 10^{10} – 10^{12} organisms/g in the colon). The intestinal microbiota plays a crucial role in the cleavage of undigested nutrients (e.g. short fatty acids), in the suppression of a pathogenic gut flora as well as in the formation of secondary BAs [7].

1.3 The enterohepatic circulation of BAs

As aforementioned, the bile is indispensable for the digestion and resorption of lipids and lipid-soluble vitamins in the small intestine. Furthermore, it plays an important role in the hepatobiliary secretion of endo- and xenobiotic metabolites such as cholesterol or drugs. The bile constitutes of BAs (mainly (un)conjugated chenodeoxycholic acid (CDCA), cholic acid (CA) and deoxycholic acid (DCA)), bilirubin, cholesterol, phospholipids and heavy metals. Due to their amphiphilic character, BAs are able to form mixed micelles together with phospholipids being important for lipid and cholesterol solubilization [8]. The primary BAs CDCA and CA are mainly synthesized from cholesterol and further processed by hepatocytes. Upon conjugation with taurine and glycine, an increased hydrophilicity and water solubility is reached. Conjugated BA molecules are subsequently excreted from hepatocytes into bile ducts, where they are stored in the gallbladder and secreted into small intestine after food intake [6]. Over 95% of the excreted BA pool is transporter-dependently reabsorbed in the terminal ileum and transported back, over the portal vein, into the liver [9]. In the colon, the remaining unconjugated BAs are transformed by bacterial enzymes into the secondary BAs DCA and lithocholic acid (LCA), and, to a smaller extent, also into ursodeoxycholic acid (UDCA). While LCA is poorly reabsorbed, DCA is easily taken up into enterohepatic circulation by passive, facilitated diffusion [8,10].

Not all hepatocytes contribute equally to BA homeostasis. Based on the distribution of key synthetic enzymes across the hepatic acinus, it has been concluded that under normal physiological conditions BA synthesis predominantly occurs in hepatocytes surrounding the central vein. In contrast, BAs that return from intestine to the liver due to enterohepatic circulation are taken up and are transported primarily by periportal hepatocytes [11].

Besides their crucial role in human physiology, BAs can enfold cytotoxic effects due to their detergent properties [12]. Abnormal BA homeostasis has been associated with general liver injury, hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), metabolic disorders including their hepatic manifestations such as non-alcoholic steatohepatitis (NASH), and

diseases of the gastrointestinal tract (e.g. inflammatory bowel disease, colorectal cancer) [8,13-15]. Hydrophobic BAs are considered to have a higher cytotoxic potential than the hydrophilic BA molecules (hydrophobicity: DCA>CDCA>CA>UDCA) [16]. The intracellular accumulation of BAs induces apoptosis or necrosis through nonspecific detergent effects and through receptor-mediated interactions of the BA molecules. BA cytotoxicity has been linked to stimulation of mitochondrial oxidative stress, generation of reactive oxygen species (ROS) and caspase activation - with secondary consequences like inflammation and fibrosis. Thereby, c-jun n-terminal kinase (JNK) activation has been discussed to play a crucial role in BA-induced cytotoxicity. Furthermore, cyclin D1 has been shown to be a relevant player in DCA-induced apoptosis in primary rat hepatocytes [17-20]. In contrast, the hydrophilic UDCA counteracts the cytotoxic BA effects - 1) by increasing the elimination of hydrophobic BAs out of the enterohepatic circulation and 2) by inhibition of cell apoptosis. UDCA is approved as a therapeutic agent against primary biliary cirrhosis and gallstones. To avoid cellular damage, BAs function as homeostatic regulators and signaling molecules to adjust their own intracellular concentrations [12]. Thereby, BA transporters and their transcriptional regulator farnesoid X receptor (FXR) are critical for the maintenance of BA homeostasis.

2. The metabolic regulator FXR

FXR is an important member of the nuclear receptor superfamily that plays a crucial role in the regulation of BA, lipid and glucose homeostasis. Furthermore, FXR is discussed to act as cell protector by promoting liver regeneration, and by elucidating antiapoptotic and anti-inflammatory effects within organs of the digestive system [21,22]. Figure 2 summarizes the most important physiological roles of FXR in the liver and intestine. The mechanisms behind are further discussed in the following chapters.

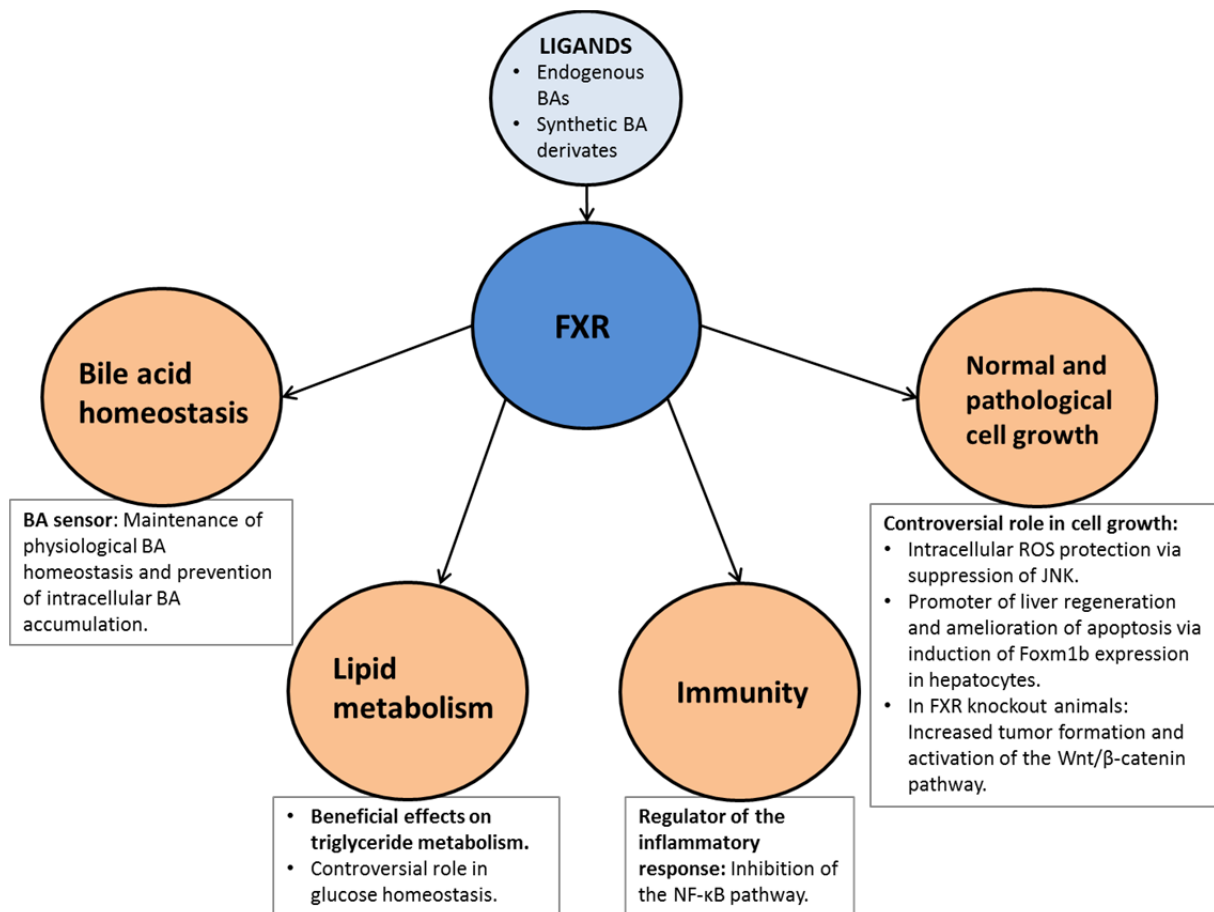


Figure 2: Physiological roles of FXR within the human digestive tract. The transcription factor FXR has been shown to be a key player in BA homeostasis, lipid metabolism, immune response, and cell growth/death regulation in the liver and intestine. BA, bile acid; Foxm1b, forkheadbox m1b; FXR, farnesoid X receptor; JNK, c-jun n-terminal kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species.

2.1 A ligand-activated transcription factor

The nuclear receptor FXR is the master regulator of BA homeostasis. It is endogenously activated by BAs to prevent intracellular BA accumulation. The hydrophobic CDCA appears to be the most efficient FXR activator, whereas DCA and CA are discussed to be partial FXR agonists [23]. The CDCA-analogical drug obeticholic acid (OCA, INT-747) is currently tested as an FXR activator and as potential therapeutic against primary biliary cirrhosis and NASH (www.intercept.com). Thereby, OCA is a 6- α -alkyl-substituted analogue of CDCA and is almost 100 times more potent with regard to FXR activation as compared to CDCA [24].

There are two known FXR genes, FXR α and FXR β , whereas the FXR β gene is a pseudogene in humans. The FXR α gene has two functional promoters and undergoes alternative splicing leading to four possible FXR protein isoforms [22]. MessengerRNA (mRNA) measurement in human tissues revealed that FXR α 1 (+/- exon 5) is predominantly expressed in the liver, whereas FXR α 2 (+/- exon 5) is the most abundant isoform in the intestine. FXR α 1 and α 2

differ in their N-terminus as a consequence of two alternative promoters [25]. FXR has a well characterized structure with a ligand-binding domain, a DNA-binding domain and two ligand-(in)dependent transactivation domains. The DNA-binding domain allows FXR to transactivate genes via binding to inverted repeat elements. The two transactivation domains are responsible for the interaction with co-regulating proteins leading to FXR transactivation or repression. FXR can transactivate genes as a monomer, but usually forms a heterodimer with retinoid X receptor (RXR) [21,26]. Table 1 summarizes the most important co-regulating proteins, ligands and epigenetic modifiers/modifications known to be involved in the regulation of endogenous FXR activity.

Table 1: Co-regulating proteins, ligands and epigenetic modifiers/modifications known to be involved in the regulation of endogenous FXR activity.

Ligands necessary for FXR-mediated transactivation	Transcriptional regulation of the FXR gene	Post-transcriptional regulation of FXR expression
Endogenous ligands <ul style="list-style-type: none"> • Bile acids • PGC1α [27] • DRIP205 [28] Synthetic ligands <ul style="list-style-type: none"> • GW4064 • INT-747 (6-ethyl-CDCA) 	Factors <ul style="list-style-type: none"> • HNF1α [29] • PGC1α-PPARγ [27] • PGC1α-HNF4α [27] Epigenetic modulation <ul style="list-style-type: none"> • CpG island at position -3.2 to -2.9 kb upstream of TSS in colon cancer [30] 	Epigenetic modulation <ul style="list-style-type: none"> • miR-421 in biliary tract cancer [31] • miR-92 in gastric cancer [32] • Sirtuin 1 [33]

CDCA, chenodeoxycholic acid; DRIP, vitamin D-interacting protein; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; PGC, peroxisome proliferator-activated receptor gamma coactivator; PPAR, peroxisome proliferator-activated receptor; TSS, transcription start site.

2.2 FXR, the BA sensor

A disturbed BA-FXR signaling axis is suggested to be important in the pathophysiology of a wide range of liver and digestive diseases such as cholestasis, hepatitis or liver and colon cancer [21]. In human physiology, BA-activated FXR promotes cellular BA efflux and detoxification, and decreases cellular uptake and synthesis via expression regulation of the involved transporters and enzymes. In other words, FXR can specifically activate genes to counteract BA toxicity. Figure 3 illustrates the enterohepatic circulation of BAs and the FXR-regulated target genes therein involved.

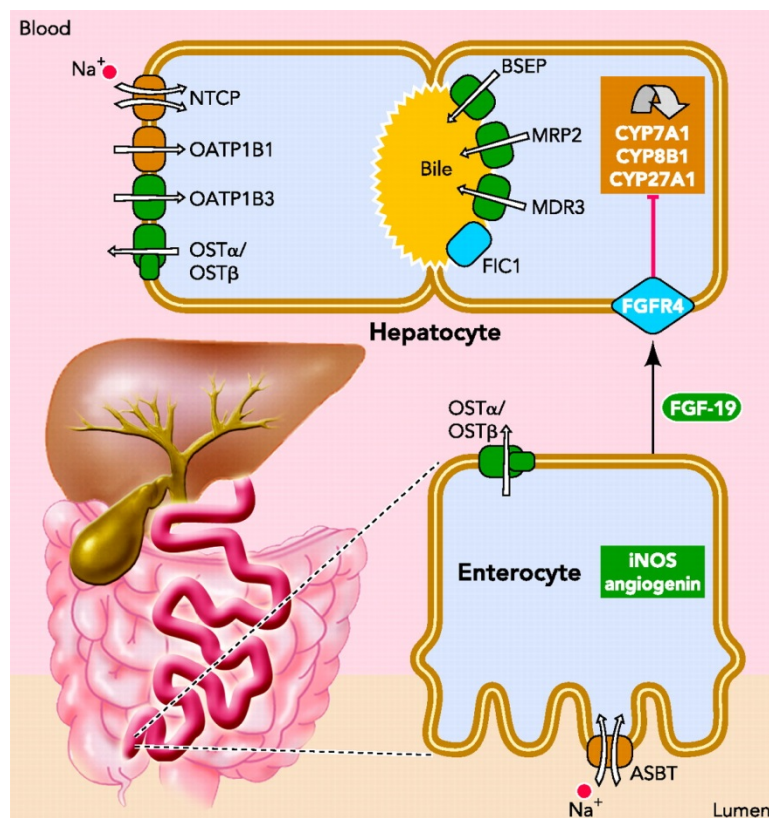


Figure 3: The enterohepatic circulation of BAs. Membrane transporters and other proteins expressed in liver and intestine, whose expression is regulated by FXR. Green: FXR-induced protein expression; Orange: FXR-suppressed protein expression. ASBT, apical sodium bile acid transporter; BSEP, bile salt export pump; CYP, cytochrome P450; FGF19, fibroblast growth factor 19; FGFR4, fibroblast growth factor receptor 4; FIC, familial intrahepatic cholestasis; FXR, farnesoid X receptor; MDR3, multidrug resistance protein 3; MRP2, multidrug resistance-associated protein 2; NOS, nitric oxide synthase; NTCP, Na^+ -taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; OST, organic solute transporter. Figure taken from [12].

Hepatocellular canalicular export of BAs is mainly mediated by the adenosine triphosphate (ATP)-binding cassette transporter bile salt export pump (BSEP). It represents the rate-limiting step of canalicular bile salt export and drives enterohepatic BA circulation [34]. Expressions of BSEP, multidrug resistance-associated protein 2 (MRP2) and multidrug resistance protein 3 (MDR3), the BA efflux transporters at the apical membrane of hepatocytes, have been shown to be directly regulated by FXR [35-37]. Whereas BSEP seems to transport mainly (un)conjugated monovalent BAs such as taurocholic acid (TCA), MRP2 is responsible for the hepatocellular export of divalent, sulphated or glucuronidated BAs into bile [38,39]. MDR3 has a critical role in translocating phospholipids across the membrane and thereby promotes mixed micelle formation counteracting BA toxicity in the biliary tree [40]. In the ileal lumen, BAs are reabsorbed by the apical sodium bile acid transporter (ASBT), the major bile salt uptake system in the ileal epithelium [12]. In the colonic lumen, remaining unconjugated BAs, particularly DCA, have been suggested to enter colonocytes by passive diffusion [13]. BA-dependent FXR activation directly induces the expression of the

heterodimeric organic solute transporter (OST) α/β , the major BA efflux transporter in the basolateral membrane of entero- and colonocytes, excreting BAs back into portal circulation [13,41,42]. OST α/β transport occurs by facilitated diffusion mechanisms and mediates cellular efflux or uptake depending on the electrochemical gradient of the substrate. Further, OST α/β is located in the basolateral membrane of hepatocytes, whereas transport activity towards BAs is negligible under physiological conditions. However in cholestatic conditions, OST α/β expression is FXR-dependently upregulated to transport BAs over the hepatic basolateral membrane back into blood circulation [18]. The BA-FXR-axis-dependent upregulation of OST α/β as an adaptive response to toxic intracellular BA accumulation has been shown to be essential in patients with primary biliary cirrhosis [43].

FXR also represses the transcription of the three BA synthesizing cytochrome P450 enzymes CYP7A1, CYP8B1 and CYP27A1 as response to intracellular BA accumulation in a negative feedback loop - in hepatocytes via upregulation of small heterodimer partner (SHP) and in enterocytes via the FGF19/FGFR4 pathway. Intestinal FXR activation induces the expression of fibroblast growth factor 19 (FGF19), which is subsequently released from the intestine into portal blood. FGF19 binds to fibroblast growth factor receptor 4 (FGFR4) on the surface of hepatocytes, thus, inducing a FGF19/FGFR4-dependent signaling cascade, which suppresses *CYP7A1* transcription in the hepatic nucleus [12,44]. SHP is a key player of repressive actions of the BA-FXR axis. The FXR/SHP pathway is also involved in the negative expression regulation of ASBT and of Na⁺-taurocholate cotransporting polypeptide (NTCP), the predominant Na⁺-dependent BA uptake transporter in the basolateral membrane of hepatocytes. NTCP is responsible for >75% of the (un)conjugated BA uptake. In contrast, the organic anion transporting polypeptides (OATP) 1B1 and 1B3 promote the Na⁺-independent hepatic BA uptake [45-47]. The human OATP family consists of 11 members, whereby OATP1B1 and OATP1B3 are the OATP transporters predominantly located at the sinusoidal membranes of human hepatocytes. Besides, OATP1B3 is also detectable in different types of cancer tissue, such as colon, liver, pancreas and lung cancer [48,49]. OATP1B1 and 1B3 are membrane transporters that are responsible for the hepatocellular uptake of a number of endogenous compounds (e.g. bilirubin, conjugated steroids and eicosanoids) and clinically important drugs such chemotherapeutics. OATP1B1 and 1B3 play an important role for the pharmacokinetics of several drugs as they precede drug activation and elimination by hepatic metabolism or biliary excretion [47,50]. OATP1B1 expression is indirectly and negatively regulated by FXR via the SHP/hepatocyte nuclear factor (HNF)-1 α pathway, whereas OATP1B3 expression is directly activated by FXR. FXR-induced OATP1B3 expression is

further discussed to maintain the hepatic extraction of xenobiotics and peptides in case of intracellular bile acid accumulation [45,51].

2.3 Lipid metabolism

Lipids are important nutrient components, which have a dietary origin or are synthesized by the liver. 95% of dietary lipids are triglycerides. Other lipids comprise phospholipids, free fatty acids, cholesterol, and fat-soluble vitamins. Dietary triglycerides are digested by enzymes into monoglycerides and free fatty acids within the gastrointestinal tract, whereas the lipid uptake into the circulatory system is facilitated by BA micelles. Endogenously, triglycerides and cholesterol are transported through the blood by (very) low and high density lipoproteins (VLDL, LDL, HDL), whereas intracellular uptake of these particles is mediated by their corresponding receptors [52]. LDL and HDL receptors are expressed in hepatic tissue in comparison to VLDL receptor, which is absent from the liver [53,54]. Impaired LDL receptor expression has been associated with hypercholesterinemia and subsequent atherosclerosis [55].

Several studies unveiled that BAs play a crucial role in maintaining lipid, glucose and energy homeostasis via the activation of proteins such as FXR or the G-protein-coupled receptor TGR5 [9,56]. Watanabe et al. showed that FXR/SHP activation plays a role in feedback regulation of hepatic fatty acid and triglyceride synthesis, and VLDL production [57]. The FXR/SHP pathway further inhibits steroid response element binding protein 1c (SREBP-1c)-mediated hepatic lipogenesis. Lipogenesis is defined as the process, where acetyl-CoA, a conversion product of glucose, is metabolized into fatty acids and subsequently into triglycerides. Triglycerides are the main constituents of human body fat [56,57]. In FXR knockout mice, hepatic lipids and circulating cholesterol and triglycerides were accumulated, whereas FXR activation by agonists decreased plasma cholesterol and triglycerides in wild-type mice [58,59]. The role of the BA-FXR signaling axis in glucose metabolism is controversially discussed in the literature. FXR activation has been associated with improved hyperglycemia in diabetic mice [59]. However, the FLINT study, which investigated the effects of the FXR activator INT-747 on adult NASH patients, revealed - besides a significant improvement in histological NASH features - an increased insulin resistance within 72 weeks of INT-747 treatment as well as elevated serum cholesterol concentrations [60]. The BA-dependent activation of TGR5 and the subsequent increased intestinal secretion of the glucagon-like peptide 1 (GLP-1) followed by enhanced insulin release from the pancreas, is assumed to be more important in glucose homeostasis as compared to FXR activation [56].

Nevertheless, activation of the BA-FXR signaling axis is still discussed as a possible treatment for metabolic syndrome and associated diseases such as non-alcoholic fatty liver disease (NAFLD).

2.4 Regulation of the inflammatory response

Although controlled inflammation is a part of the biological immune response to harmful stimuli in human tissues, an uncontrolled and chronic inflammation is a crucial and feared mechanism that is involved in the progression of many liver and digestive diseases such as NAFLD, viral hepatitis, drug-induced liver injury or inflammatory bowel disease. Preceding chronic tissue inflammation has been linked to cancer development throughout the whole human digestive system. After lipopolysaccharide (LPS) administration to FXR knockout mice, the murine hepatic tissue developed massive necrosis and inflammation, whereas no significant liver damage could be observed in wild-type animals. Mice with constitutively hepatic expression of FXR after adenoviral VP16-FXR tail injection showed decreased inflammatory response to LPS injection. In this context, it could be demonstrated that FXR is a negative modulator of nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B)-mediated hepatic inflammation [61]. NF- κ B is a nuclear transcription factor that enhances the expression of inflammatory markers such as tumor necrosis factor (TNF) α or interleukin (IL)-1 β [21]. Excessive intracellular BA accumulation has been linked to the activation of the NF- κ B signaling pathway in liver and colon cells, showing more uncontrolled liver inflammation in FXR knockout mice as compared to wild-type animals. These findings confirm the hepatoprotective effects of FXR regarding uncontrolled activation of inflammatory processes [62,63].

2.5 Impact on normal and pathological cell growth

The exact role of FXR in cell growth regulation, apoptosis and carcinogenesis is still not fully elucidated. The published literature regarding hepato- and intestine-protective effects of FXR in cancerogenesis are mainly based on FXR-knockout animal studies. Kim et al. for example showed that FXR-null mice exhibited elevated serum and hepatic BA levels, an increased inflammatory response and a higher incidence of hepato(cholangio)cellular carcinoma [64]. In mouse intestine, loss of FXR expression and subsequent elevations of intestinal BA levels led to earlier mortality caused by increased tumorigenesis. Unlike in the liver, intestinal FXR expression loss itself seemed to increase tumor susceptibility and not merely the subsequently elevated BA concentrations [65]. FXR is assumed to be a direct suppressor of the Wnt/ β -

catenin pathway leading to decreased cyclin D1 expression [66]. FXR is further discussed to act as a suppressor of the JNK/c-jun pathway via suppression of ROS production [67]. In contrast, pathways involving increased cyclin D1 and c-jun expressions are also debated as promoters of apoptotic processes, dependent on concomitant signals (e.g. BA-mediated; q.v. para. 1.3) and the cell type [68-70]. These findings are in line with reports showing that FXR activation reduces inflammatory, apoptotic and fibrotic processes in NASH [71]. Further, FXR activation improved defective regeneration in aging mouse livers via FXR-dependent induction of forkheadbox m1b (Foxm1b) expression. Foxm1b is a proliferative transcription factor required for normal liver regeneration, which is inversely correlated with apoptosis and is highly upregulated in human HCC as compared to non-tumorous livers [21,72,73]. These cited studies highlight the ambivalent role of FXR in cell growth regulation and further research is necessary to elucidate the real role of FXR in carcinogenesis.

3. MicroRNAs and their regulatory role in the human digestive system

3.1 Epigenetic, post-transcriptional regulators of gene expression

Besides DNA methylation and histone modification, microRNAs (miRNAs, miRs) belong to the epigenetic regulatory network, which coordinates the expression of the human genome. It has been estimated that expression of 60% of all human genes are miRNA-dependently regulated [74]. To date, the miRBase database lists 1881 human precursor miRNA sequences (www.mirbase.org). MiRNAs are short non-coding RNA molecules of 18–25 nucleotides in length, which regulate target gene expression on a post-transcriptional level [75,76]. Figure 4 illustrates the endogenous formation of mature miRNA molecules. Every miRNA molecule contains a seed sequence (nucleotides 2-7) that particularly matches with sites located within the 3' untranslated region (UTR) of a mRNA [74]. Usually, only one mature miRNA strand is formed *in vivo*, whereas the other strand of the miRNA duplex molecule is degraded. But, at least 80 different human miRNA precursors can yield two abundant, functional relevant, mature miRNAs - a 5' strand (-5p) and a 3' strand (-3p) with different seed sequences and mRNAs as binding targets. However, there is increasing evidence for interplay between the 5' and the 3' strand arm of the same precursor molecule targeting the same group of genes and reinforcing one certain phenotype [77]. The pleiotropic effects of miRNA molecules, meaning their ability to regulate the expression of a large set of target genes in parallel, illustrate a currently discussed problem in the consideration of miRNAs as potential therapeutics.

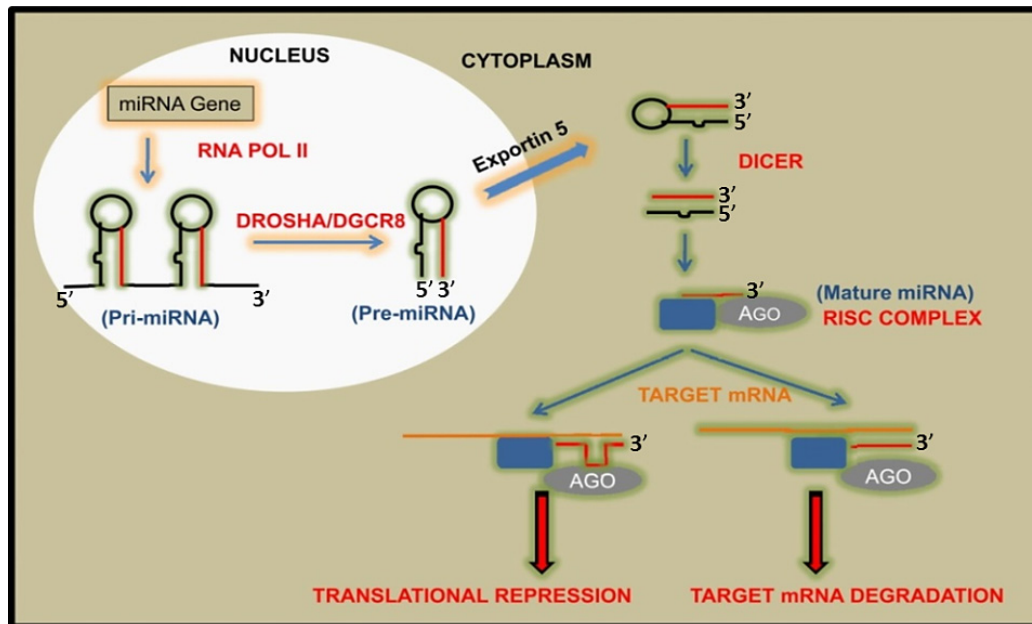


Figure 4: Biogenesis and function of miRNAs. Almost all miRNA genes are transcribed by the RNA polymerase II into pri-miRNAs, which are long transcripts including multiple hairpin loop structures. Pri-miRNAs are further processed into pre-miRNAs by the Microprocessor protein complex containing the RNase Drosha. Pre-miRNAs are precursor molecules of 70-80 nucleotides in length that contain a characteristic hairpin loop structure. These precursors are then further exported from the nucleus into cytoplasm by exportin 5. There, pre-miRNAs are processed by the RNase Dicer into short, double-stranded miRNAs, which are then converted to mature, single-stranded miRNAs via RNA-induced silencing complex (RISC). RISC is a protein complex that contains argonaute (AGO) proteins. Finally, mature miRNAs direct RISC to target mRNAs, leading to their degradation (when perfect complementary binding) or translational repression (when imperfect complementary binding). AGOs are the catalytic enzymes with endonuclease activity that are responsible for target mRNA degradation. Adapted from [78].

3.2 Physiological and pathological roles

About 1400 human mature miRNAs are described in the literature, for which sufficient evidence exists for an important role in normal human development, cell differentiation and cell growth control [79]. MiRNA molecules are already entering the clinic as diagnostic and prognostic biomarkers and further as therapeutic targets and agents referring to several diseases of the human digestive system such as (steato)hepatitis, cholestasis, drug-induced liver injury, (cholangio)hepatocellular carcinoma, cholangiocarcinoma, colon cancer or inflammatory bowel disease. The (patho)physiological role of miRNAs in the regulation of key-cancer pathways such as cell cycle control is currently mostly discussed in the literature. In this context, the liver-relevant miR-192 and miR-34a have reached attention because of their ability to interact with the tumor protein p53 [79]. p53 (*TP53*) has been dubbed the “guardian of the genome” that mediates apoptosis and tumor suppression [80]. The transcription factor p53 is the most frequently mutated gene in human cancers, whereby mutations often lead to a reduced or completely abolished transcriptional function of this protein [81]. Pichiorri et al. provided evidence that miR-192 is transcriptionally activated by

p53, thus, modulating the expression of the mouse double minute 2 homolog (MDM2), a negative regulator of p53 [82]. Alternative miRNAs have further been described to influence this MDM2/p53 feedback loop such as miR-509 or miR-122-3p [83,84]. Comparable to the miR-192/MDM2/p53 interaction, a similar interplay has been shown for miR-34a. p53 can activate the transcription of miR-34a, which in a consequence leads to a suppression of nicotine adenine dinucleotide-dependent deacetylase sirtuin-1 (SIRT1) expression. Through this mechanism, decreased SIRT1-dependent deacetylation of p53 is leading to an increased p53 function, but, however, also to more acetylated FXR [33,79]. Acetylation of FXR increases its stability, but inhibits its heterodimerization with RXR α and transactivation ability. However, SIRT1 overexpressing mice, which anamnestically underwent partial hepatectomy, revealed decreased FXR, SHP and BSEP protein expressions [85]. Based on this finding, other SIRT1-dependent epigenetic modifications such as histone deacetylation, may influence FXR activity and conclusions about any functional relevance of the miR-34a/SIRT1 interplay on FXR activity are currently difficult to draw.

There are further target mRNAs that are described to be (in)directly regulated by miR-192 or miR-34a. The ones, which play a relevant role in cell cycle regulation of liver and intestinal cells, are summarized in Figure 5.

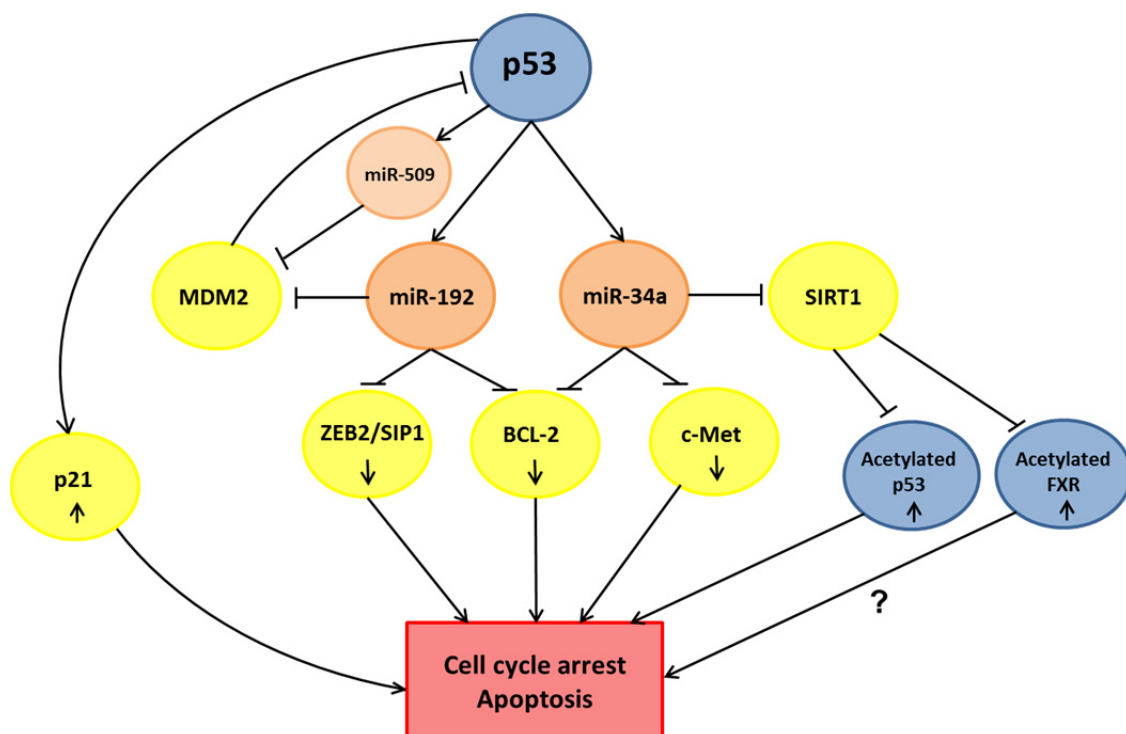


Figure 5: (In)direct target mRNAs of the p53-regulated miRNAs -192, -509 and -34a, which play a crucial role in cell cycle arrest and apoptosis. All three miRNAs form a positive feedback loop with the endogenous cell cycle regulator p53. BCL-2, B-cell lymphoma 2; FXR, farnesoid X receptor; MDM2, mouse double minute 2 homolog; SIRT1, sirtuin 1; ZEB2/SIP1, zinc finger E-box binding homeobox 2/Smad interacting protein 1; [33,82,86-89].

4. Transcriptional and miRNA dysregulation in hepatic and intestinal diseases

A disturbed BA-FXR signaling axis and shifts in expression of p53-dependently regulated miRNAs are both discussed in the literature to play a pivotal role in diseases of the human digestive system, where mainly cell cycle regulatory processes are pathologically involved. Figure 6 gives an overview of known causes promoting the progression of different liver diseases, including changed miRNA expression levels and a disturbed BA-FXR signaling axis. As such alterations are additionally well described for colon carcinogenesis, we will focus in the next paragraphs on FXR-/miRNA-dependent pathomechanisms involved in cholestasis, NAFLD, liver and colon cancer.

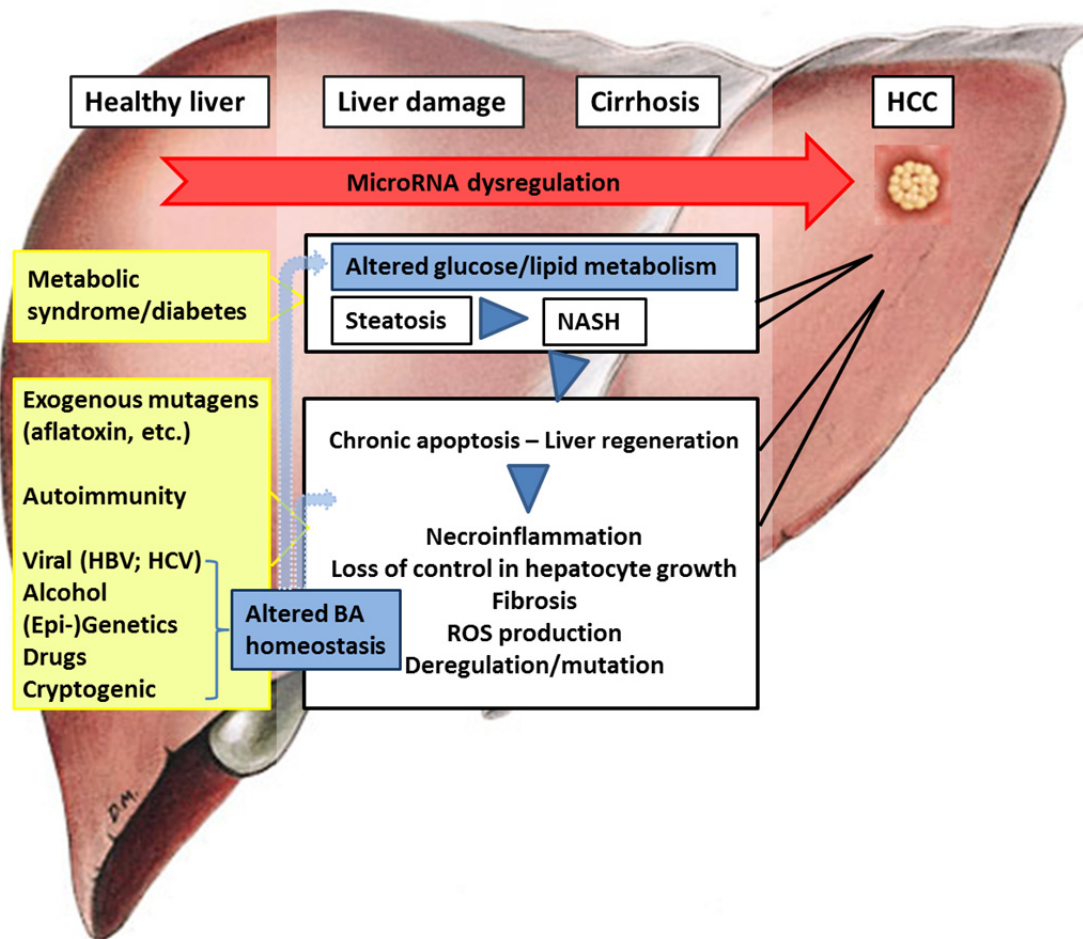


Figure 6: Summary of known causes leading to liver damage, cirrhosis and liver cancer development. In all liver disease-stages changed expression levels of p53-controlled miRNAs (e.g. miR-192, miR-34a) or an altered BA-FXR signaling axis are described to promote disease progression. An imbalance between apoptosis and liver regenerative processes can promote liver inflammation leading to uncontrolled hepatocyte growth, formation of excess fibrous connective tissue, oxidative stress, and subsequent irreversible cirrhotic liver and/or tumorigenesis. Yellow: known causes of non-alcoholic liver disease (NAFLD), hepatitis, cholestasis or other liver injuries. BA, bile acid; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species. Adapted from [90].

4.1 Cholestatic liver

Cholestasis is characterized by impaired bile secretion and/or bile flow between liver and duodenum, which leads to an accumulation of BAs in hepatocytes and the intrahepatic bile duct system (intrahepatic cholestasis) or in the extrahepatic biliary tract (extrahepatic cholestasis). Intracellular hepatic BA accumulation can promote inflammation, fibrosis, cirrhosis and subsequent tumorigenesis in the liver (Fig. 6). The most important hepatic, compensatory anticholestatic response is the BA-dependent FXR activation and the subsequent changes in BA homeostasis (q.v. para. 1.2.2). It has been hypothesized that a decreased BA level in the intestinal lumen leads to an insufficient intestinal FXR/FGF19 response, which induces an inappropriate hepatic BA neosynthesis and progressive liver damage in cholestatic patients [21,91]. Rare mutations in genes coding for BA transporters located in the canalicular membrane of hepatocytes are known that result in intrahepatic cholestasis: e.g. progressive familial intrahepatic cholestasis PFIC2 due to an impaired function of BSEP [92]. Genetic variations in *NR1H4*, the gene coding for FXR, were associated with intrahepatic cholestasis of pregnancy [93]. Until now, no studies are available, which investigate the miRNA-dependent regulation of FXR expression in the context of BA homeostasis. Instead, miR-33 has been observed to regulate the expression of BSEP and elevated miR-33 levels have been shown to have a functional relevance by disturbing the canalicular BA secretion in mice [94]. Furthermore, upregulation of miR-34a and the subsequent impact on SIRT1 expression has been associated with BA-induced tissue injury [95] based, however, on NAFLD rat model studies [96]. Lee et al. observed that FXR suppresses miR-34a expression via binding of SHP to its promoter, which thereby inhibits promoter occupancy of p53 [33]. But there are no studies in the literature, which reveal a direct link between changed miR-34a expression levels and intrahepatic cholestasis formation. Although not yet sufficiently investigated, we hypothesize that miRNAs play a key role in the maintenance of BA homeostasis and in the pathophysiology of intracellular BA accumulation, i.a. via their interactions with FXR.

4.2 Non-alcoholic fatty liver disease

NAFLD is a common and increasing cause of chronic liver disease in the Western world, affecting up to 30% of all Americans [97]. NAFLD is characterized by an abnormal accumulation of triglycerides in hepatocytes leading to liver steatosis in the initial stage [6]. NASH is characterized by hepatic inflammation, which promotes the development of fibrotic liver tissue [97]. NASH progresses in 15–20% to cirrhosis. It is a rising indication for liver

transplantation and is associated with an increased risk for HCC. Obesity, diabetes, metabolic syndrome and insulin resistance are risk factors leading to NAFLD [21,97]. At present, there are no pharmacological treatments approved for NASH [60,98]. When bound to FXR, lipophilic BAs reduce circulating triglycerides levels due to inhibition of lipogenesis. Besides stimulation of FXR activity, BA-dependent activation of TGR5 is further discussed as possible pharmacological target for the treatment of NAFLD (q.v. para. 2.3) [56,60,99]. In addition to the FXR ligand INT-747, a BA-modified dual FXR/TGR5 agonist (INT-767) is currently investigated as a potential treatment for NAFLD-associated fibrosis (www.interceptpharma.com).

Histological features of cholestasis are not typical for NAFLD, which suggests the co-existence of another liver disease if present. However, there are several mouse and human NAFLD studies showing that a concomitant dysfunction of the FXR/SHP pathway results in moderately increased BA levels and promotes the inflammatory cascade and the progression of NASH [21,100-103]. Aranha et al. showed that hepatic DCA, CDCA and CA levels were moderately elevated by 92, 64, and 43%, respectively, in patients with steatohepatitis as compared to healthy controls. Furthermore, significant correlations were found between hepatic CDCA levels and fibrosis in NASH patients conferring an association between specific BAs and disease progression, possibly through BA-induced liver injury [104].

As JNK-dependent hepatocyte lipoapoptosis is claimed to be a key feature in NAFLD [105], the potential involvement of apoptosis-related miRNAs has to be highlighted here. In mouse models and humans, an elevated expression of miR-122, miR-192 and miR-34a are discussed as potential serum biomarkers in NAFLD [106]. In steatosis, hepatic miR-192 and miR-122 expression levels were increased as compared to healthy liver tissue and as compared to tissue obtained from NASH patients. Pirola et al. claimed that the overall miR-192/-122 expression is elevated in NAFLD. With progression of liver injury, an increased release of these two miRNAs into serum was observed [107]. Beside chronic inflammatory processes, enhanced miR-192 expression was further associated with transforming growth factor (TGF)- β /Smad signaling-driven fibrosis [108]. Also hepatic miR-34a expression increased, SIRT1 expression decreased and the levels of acetylated p53 progressively rose with NAFLD disease severity in obese patients [96]. In fatty liver of obese mice, a disturbed FXR/SHP pathway was associated with the increased miR-34a expression levels [33]. In line with these observations, Panasiuk et al. showed that intensification of NAFLD-associated inflammation induces the cell cycle regulator p53 [109]. Based on these findings we hypothesize that the p53-regulated

miRNAs miR-34a and miR-192 as well as a disturbed FXR/SHP pathway play a crucial role in the progression of NAFLD.

4.3 Malignancies

4.3.1 Hepatocellular carcinoma

Malignant tumors of the liver are classified in primary and secondary (metastatic, e.g. originated from colorectal cancer) malignancies. HCC, a cancer formed by hepatocytes, is accounting for approximately 75% of all primary liver cancers. 6% of primary liver malignancies are cholangiocarcinoma, originating from the intrahepatic bile ducts [110]. As shown in Figure 6, HCC usually develops on the basis of abundant chronic liver diseases. Main causes are chronic hepatitis B and C, but HCC can also be the end stage disease of cholestatic liver or non-viral hepatitis. The link between cholestatic liver disease and liver cancer has already been established in clinical studies. E.g. children with mutated BSEP suffering from PFIC2 were prone to liver tumor formation [111]. Furthermore, there is increasing evidence that NAFLD is frequently underlying liver cancer development [112]. HCC is as a very aggressive tumor and often diagnosed late in its course because of the absence of pathognomonic symptoms. Additionally, HCC is considered to be a relatively chemotherapy-refractory tumor, with sorafenib as the established standard monotherapy. Platin derivatives are used as second-line therapy. Consequently, HCC is the second leading cause of cancer-related death in men [113].

As aforementioned, the role of FXR in carcinogenesis is still not clear. However, several authors discuss a cancer-protective role of FXR based on results obtained from FXR-null animal models. Additionally, several studies showed reduced FXR expression in human HCC tissues as compared to non-tumorous tissue [114,115]. In contrast, an immunohistochemical study observed a preserved or enhanced FXR protein expression in tumor cell nuclei of human HCC tissue compared with hepatocyte nuclei of normal and diseased liver [116].

Hundreds of epigenetic pathways, including miRNAs, are described in the literature to be disturbed in HCC. The high number of published *in vitro* and *in vivo* studies illustrates the complicated network underlying carcinogenesis. Currently, miR-34 mimic replacement therapy (MRX34) is investigated in phase I drug development stage as the first miRNA-based treatment against primary and secondary liver cancers [117]. The p53/miR-34 pathway is discussed to be involved in the development of different cancers such as liver and colon tumors, showing a downregulated miR-34a expression as compared to non-tumorous tissue. *In vitro* studies performed in liver and colon cancer cell lines showed a miR-34a-dependent

suppressive effect on cell growth, invasion and migration [88,118]. Although, miR-192 is not considered to be typically changed in expression in several studies on HCC, Lian et al. observed a significantly suppressed expression of miR-192 in HCC tissue as compared to non-tumorous tissue [119].

4.3.2 Colon carcinoma

Most of the malignant, polypoid-ulcerating intestinal tumors are adenocarcinoma, originating from glandular cells of the intestinal mucosa [6]. Colorectal cancer (CRC) is the fourth leading cause of cancer-related death in men. In the last years, a trend towards decreased mortality can be attributed to more CRC screening and improved cancer prevention and treatments. Effective, adjuvant standard therapeutics are for example platin derivates such as oxaliplatin and 5-fluorouracil in combination with folinic acid, or irinotecan. Risk factors for developing CRC are of genetic and environmental origin such as obesity, physical inactivity and a high consumption of red and processed meat [113]. Furthermore, there is a well-documented association between inflammatory bowel disease and colonic neoplasia. It has been claimed that high fat diet modifies the progression of colon cancerogenesis by increasing the amount of serum and fecal BAs. Particularly, DCA and LCA have been strongly associated with colon tumorigenesis as they affect the colonic epithelium via promotion of DNA oxidative damage, inflammation, and enhanced cell proliferation [13,21,120]. In healthy tissues, FXR expression gradually increases from terminal ileum to the sigmoid colon. In colon carcinoma, FXR levels are reduced as compared to peritumoral nonneoplastic mucosa and were inversely correlated with tumor stage [121]. In mouse intestine, knockout of FXR expression resulted in elevated intestinal BA concentrations, an increased inflammatory response and earlier mortality because of increased tumor progression [65].

MiR-192 is hypothesized to be a risk biomarker for colon cancer-originated liver metastasis as well as a high-potential drug agent for primary colon cancer [87]. Loss of p53 function by mutation and consequently decreased miR-192 and miR-34a expressions might play a crucial role in colon cancerogenesis [122,123]. Similarly to liver cancer, several hundred miRNAs are described in the literature to be involved in colon tumorigenesis, which underlines the need for more human studies investigating the real endogenous role of certain miRNAs in liver and colon cancerogenesis.

5. Objectives of the thesis

Little is known about the bidirectional interaction between the BA sensor FXR and miRNAs in diseases of the human digestive system. The overall aim of this thesis was to investigate changes in the functionality of the BA-FXR axis based on shifts in miRNA expression and the impact of this interplay on metabolic pathways and cell cycle regulation. We aimed to study if FXR and FXR target genes are epigenetically regulated by miRNAs and, as a consequence, if a disturbed BA-FXR axis might influence the endogenous miRNA network *in vitro* and *in vivo*. By elucidating the miRNA-dependent epigenetic pathways involved in the regulation of endogenous FXR activity, opportunities for potential novel therapeutics against liver and intestinal diseases shall be discussed.

6. Structure of the thesis

In Chapter 1 we demonstrate that BA-FXR-controlled signaling influences the endogenous miRNA network involved in metabolic and cell cycle regulation-associated pathways of the liver. CDCA-dependent activation of the BA-FXR signaling axis significantly impacts the expression of miRNAs and genes involved in lipid, BA and drug metabolism in primary human hepatocytes. Furthermore, this chapter illustrates a link between the apoptotic, p53-regulated miRNA miR-34a and BA homeostasis in dependence of FXR transactivation.

In Chapter 2 we show that FXR and FXR target genes, all of which are important in the maintenance of BA homeostasis, are epigenetically regulated by the p53-regulated miRNA miR-192. Besides, the cell cycle-regulatory role of the miR-192/FXR interplay on proliferation of liver and colon cancer cells is elucidated.

In Chapter 3, an additional direct miRNA-dependent expression regulation of the FXR target gene OATP1B3 is demonstrated. This chapter highlights the complexity of the endogenous miRNA gene regulatory network by showing a “multiple-to-one” relationship between miRNAs and OATP1B3 expression.

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II. CHAPTER 1

Chenodeoxycholic acid significantly impacts the expression of miRNAs and genes involved in lipid, bile acid and drug metabolism in human hepatocytes

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Chenodeoxycholic acid significantly impacts the expression of miRNAs and genes involved in lipid, bile acid and drug metabolism in human hepatocytes

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ABSTRACT

Aims: Bile acids (BAs) are important gut signaling hormones, influencing lipid, glucose, and energy homeostasis. The exact mechanisms behind these effects are not yet fully understood. Lately, they have come to the fore as putative therapeutics in metabolic diseases, such as e.g. nonalcoholic fatty liver disease (NAFLD). We elucidate to what extent BAs impacts on the mRNAome and microRNAome in hepatocytes to gather novel insights into the mechanisms behind metabolic and toxicologic effects of bile acids.

Main methods: Five batches of primary human hepatocytes were treated with 50 μmol/l chenodeoxycholic acid (CDCA) for 24 or 48 h. Total RNA was extracted, size fractionated and subjected to Next Generation Sequencing to generate mRNA and miRNA profiles.

Key findings: Expression of 738 genes and 52 miRNAs were CDCA dependently decreased, whereas 1566 genes and 29 miRNAs were significantly increased in hepatocytes. Distinct gene clusters controlling BA and lipid homeostasis (FGF(R), APO and FABP family members, HMGCS2) and drug metabolism (CYP, UGT and SULT family members) were significantly modulated by CDCA. Importantly, CDCA affected distinct microRNAs, including miR-34a, -505, -885, -1260 and -552 that systematically correlated in expression with gene clusters responsible for bile acid, lipid and drug homeostasis incorporating genes, such as e.g. *SLC01B1*, *SLC22A7*, *FGF19*, *CYP2E1*, *CYP1A2*, *APO* family members and *FOXO3*.

Significance: Bile acids significantly modulate metabolic and drug associated gene networks that are connected to distinct shifts in the microRNAome. These findings give novel insights on how BA enfold metabolic and system toxic effects.

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Abbreviations: ABC, ATP-binding cassette transporter; AGPAT2, 1-acylglycerol-3-phosphate O-acyltransferase; Ahr, arylhydrocarbon receptor; AKR1C1, aldo-keto reductase 1C1; APO, apolipoprotein; ASBT, apical sodium dependent bile acid transporter; BAs, bile acids; BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; CPT1A, carnitine palmitoyltransferase 1A; CYP, cytochrome P450; DM, drug metabolism; DMSO, dimethyl sulfoxide; FABP, fatty acid-binding protein; FASN, fatty acid synthase gene; FDR, false discovery rate; FXR, farnesoid X receptor; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; LDLR, low density lipoprotein receptor; mRNA, messenger RNA; miRNA, microRNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NPC1L1, Niemann-Pick C1-like 1 gene; NTCP, Na⁺-taurocholate cotransporting polypeptide; OATP, organic anion transport protein; OST, organic solute transporter; PCR, polymerase chain reaction; PHHs, primary human hepatocytes; PPAR-γ, peroxisome proliferator-activated receptor; RT-PCR, real-time PCR; S1PR, sphingosine-1-phosphate receptor; SHP, small heterodimer partner; SLC, solute carrier family; STARD3, StAR-related lipid transfer domain protein 3; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; UTR, untranslated region.

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1. Introduction

Bile acids (BAs) are important endogenous compounds responsible for the efficient absorption of lipid-soluble compounds in the intestine. Semiquantitative BA derivatives, such as e.g. obeticholic acid (OCA), are currently discussed as future treatment option for different metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD), the most common liver disease in the western world [1]. There is an urgent need to better understand how BAs enfold their effects on metabolic pathways and on their own homeostasis to better estimate safety and efficacy of these compounds.

The effects of BAs on metabolism are triggered by their interaction with the nuclear receptor FXR, which leads to improvement of steatosis and fibrosis in NAFLD [1,2] and a bettering of hepatic insulin sensitivity [3]. By interacting with FXR, BAs also regulate their own homeostasis via negative and positive feedback loops [4], thus preventing cells from an

Table 1
Clinical characteristics of liver cell donors.

Patient No	Gender	Age	Main diagnosis	drugs
1	Female	Between 71 and 80	Colon carcinoma and liver metastases	None
2	Female	Between 70 and 79	GIST tumor	None
3	Female	Between 51 and 60	Rectosigmoid carcinoma with liver metastases	State after 6 cycles of oxaliplatin/folinic acid(FUFOX)
4	Female	Between 51 and 60	Liver metastasis after kidney cancer	Avastatin Bisoprolol L-thyroxine Zopiclone Anagrelide
5	Female	Between 31 and 40	Hepatocellular carcinoma	Metamizole

intracellular BA overload with toxic effects [5]. The enterohepatic circulation and homeostasis of BAs are ensured by a coordinated action of BA uptake and efflux transporters. Bile acid transporters include amongst others the apical sodium-dependent bile acid transporter (ASBT) and organic solute transporters α/β (OST α/β) in the intestine, and the Na⁺-taurocholate co-transporting polypeptide (NTCP), the organic anion-transporting polypeptides OATP1B1 and OATP1B3 and the bile salt export pump (BSEP) in hepatocytes [6].

As ligands for the nuclear receptors FXR, PXR or CAR, BAs regulate the expression of several genes that are important for both BA homeostasis and drug metabolism, such as e.g. OATP1B1, OATP1B3 and CYP3A4. This effect may give space for interactions between BAs and therapeutics and may affect drug exposure margins with consequences for drug safety and efficacy. By interacting with several other receptor molecules, besides FXR, such as muscarinic receptors or G protein-coupled receptors (i.e. TGR5) [7,8], BAs influence lipid and energy homeostasis. TGR5 triggers weight loss upon activation by BAs [9–11] and has a positive impact on glucose tolerance by inducing the secretion of glucagon-like-peptide 1 (GLP-1) from intestinal enteroendocrine cells [12].

Notwithstanding the role of genetic susceptibility factors in the pathogenesis of obesity and diabetes [13–16], the complex interplay of genetically, epigenetically and microRNA (miRNA)-driven factors is becoming increasingly evident [17–20]. In particular, shifts in the concentrations of miRNAs, small noncoding molecules that inhibit mRNA

translation, can lead to rapid changes in protein expression [21]. MiRNAs belong to a relatively small pool of molecules controlling the expression of major parts of the genome. Thus, expression changes of only few miRNAs can broadly impact the functional integrity of different metabolic and signaling pathways.

In the current study we investigate the effect of the BA chenodeoxycholic acid (CDCA) on the expression of the miRNAome and mRNAome in primary human hepatocytes (PHHs) and assess to what extent CDCA induces systematic shifts in gene networks responsible for BA and lipid homeostasis as well as drug metabolism. BA-induced shifts in the miRNA profile are set in context to the investigated networks to elucidate novel miRNA driven regulatory pathways influencing the expression of the mentioned gene networks.

2. Material and methods

2.1. Primary human hepatocytes (PHH)

The study was approved by the Ethics Committee of the Canton of Zurich, Switzerland (study number EK-680) and the Human Tissue and Cell Research (HTCR) Foundation. The HTCR-process that included written informed consent was approved by the Ethics Committee of the Medical Faculty of the Ludwig Maximilians University (approval number 025-12) and complied with the Bavarian Data Protection Act. PHH were obtained from five patients in Germany who were



p-value	FDR	No of objects*	Network Objects
0.000000	0.000075	7/42	SHP, MDR1, OST alpha/beta, MDR3, BSEP, OSTalpha, OSTbeta
0.000002	0.000155	6/35	SULT1E1, CYP3A4, UGT1A10, CYP2C8, CYP1A1, CYP1A2
0.000002	0.000155	6/35	SULT1E1, CYP3A4, UGT1A10, CYP2C8, CYP1A1, CYP1A2
0.000002	0.000163	6/37	SULT1E1, CYP3A4, UGT1A10, CYP2C8, CYP1A1, CYP1A2
0.000007	0.000348	7/66	CYP4A11, CYP3A4, UGT1A8, CYP2C8, CYP2E1, CYP1A1, CYP1A2
0.000010	0.000431	7/70	CYP4A11, CYP3A4, UGT1A8, CYP2C8, CYP2E1, CYP1A1, CYP1A2
0.000289	0.010765	4/30	HMGCS2, SHP, RARalpha, ADHG
0.000329	0.010765	4/31	FGF19, SHP, CYP3A4, CYP7A1
0.000529	0.015403	4/35	UGT1A9, AKR1C1, UGT1A8, UGT1A10
0.000590	0.015467	4/36	UGT1A9, AKR1C1, UGT1A8, UGT1A10

Fig. 1. Gene enrichment analysis showing signaling pathways most affected by CDCA.

undergoing liver resection because of liver metastases in association with primary tumors in colon or kidney or because of hepatocellular carcinoma. The clinical characteristics of the patients are summarized in Table 1 (supplementary information). PHHs were prepared as earlier described [22] and kept in six-well plates in hepatocyte maintenance medium supplemented with UltraGlutamine for approximately 5 h before further treatment procedures. PHHs were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ at atmospheric pressure.

2.2. Cell treatment and whole RNA isolation

PHHs of 5 patients were kept in 6 well plates. 24 h later duplicate wells of each cell batch were treated with chenodeoxycholic acid (CDCA) or dimethyl sulfoxide (DMSO) (vehicle control) (both from Sigma–(both from Sigma-Aldrich, Buchs, Switzerland). One CDCA and one DMSO treated well of each cell batch were harvested together 24 h or 48 h after cell treatment using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for combined DNA and RNA isolation. Subsequently, total RNA was purified using the miRNeasy kit from Qiagen (QIAGEN, Hombrechtikon, Switzerland).

Table 2

Effect of CDCA on the expression of genes involved in bile acid homeostasis and drug metabolism in primary human hepatocytes after 48 h.

Gene name*	log ₂ ratio	Fold change	p-Value	FDR
<i>Up-regulated genes</i>				
<i>Bile acid homeostasis</i>				
FGF19	5.078	33.78	3.541xE-07	9.624xE-05
SLC51B	4.934	30.57	1.091xE-15	2.958xE-12
NR0B2	2.85	7.21	9.207xE-11	8.158xE-08
ABCB11	2.195	4.58	2.194xE-09	1.177xE-06
SLC51A	2.194	4.58	8.15xE-16	2.768xE-12
ABCB4	1.603	3.04	5.099xE-10	3.149xE-07
ABCG5	0.8736	1.83	0.0015	0.065
SLCO1B3**	0.5981	1.51	0.0045	0.124
ABCG8	0.4935	1.41	0.0438	0.434
<i>Drug metabolism</i>				
UGT2B10	1.264	2.4	0.0006	0.033
SULT1C2	1.13	2.19	0.0393	0.412
UGT1A8	0.9499	1.93	0.021	0.298
UGT2B4	0.7462	1.68	0.0098	0.196
UGT1A3	0.5976	1.51	0.0269	0.34
UGT1A1	0.5671	1.48	0.0416	0.425
PPARG	0.5001	1.41	0.0431	0.432
<i>Down-regulated genes</i>				
<i>Bile acid homeostasis</i>				
EPHX1**	−0.4618	0.73	0.035	0.389
NR1I3	−0.5675	0.67	0.0106	0.207
SLCO1B1	−0.5869	0.67	0.0046	0.126
BAAT	−0.8562	0.55	0.0035	0.107
CYP3A4**	−1.734	0.3	2.055xE-13	3.49xE-10
CYP7A1	−6.271	0.01	9.197xE-18	4.686xE-14
<i>Drug metabolism</i>				
AHR	−0.4569	0.73	0.0413	0.423
UGT1A6	−0.5588	0.68	0.0127	0.229
NR1I3	−0.5675	0.67	0.0106	0.207
SLCO1B1	−0.5869	0.67	0.0046	0.126
UGT2A3	−0.7303	0.6	0.0026	0.09
SULT1B1	−0.7455	0.6	0.0017	0.067
UGT1A7	−0.9585	0.51	0.0423	0.429
SLC22A7	−1.054	0.48	0.0189	0.282
CYP2C8	−1.605	0.33	8.14E-07	<0.001
SULT1E1	−1.679	0.31	0.0014	0.063
SLC22A1	−1.726	0.3	8.55E-12	1.09E-08
CYP1A2	−1.921	0.26	1.05E-07	3.23E-05
CYP1A1	−1.954	0.26	1.96E-05	0.002
UGT1A9	−2.095	0.23	6.84E-05	0.007
CYP2E1	−2.556	0.17	2.03E-21	2.07E-17

* Genes involved in drug metabolism or bile acid homeostasis with a FDR < 0.46 and p-value < 0.05.

** Involved in both bile acid homeostasis and drug metabolism or drug transport.

2.3. Next generation sequencing

For library preparation the quality of the isolated RNA was evaluated using a Qubit® (1.0) Fluorometer (Life Technologies, Carlsbad, CA, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only those samples with a 260 nm/280 nm ratio between 1.8 and 2.1 and a 28S/18S ratio of 1.5–2 were further processed. 3' and 5' RNA adapters were ligated to total RNA samples (1 µg) using the TruSeq small RNA Sample Prep Kit v2 (Illumina, Inc., San Diego, CA, USA). Ligated samples were reverse-transcribed into double-stranded cDNA and fragments containing TruSeq adapters on both ends were selectively enriched by polymerase chain reaction (PCR). The small RNA fraction (145–160 bp) was selected and isolated by polyacrylamide gel electrophoresis. The quality and quantity of the enriched libraries were validated using a Qubit® (1.0) Fluorometer and the Caliper GX LabChip® GX (Caliper Life Sciences, Inc., Hopkinton, MA, USA). The libraries were diluted to 10 nM in Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20. For cluster generation and sequencing the TruSeq PE Cluster Kit v3-cBot-HS was used with 10 pM of pooled normalized libraries on the cBOT system (Illumina, Inc.). Sequencing was performed on the Illumina HiSeq 2000 using the TruSeq SBS Kit v3-HS (Illumina, Inc.).

Table 3

Effect of CDCA on the expression of genes involved in lipid homeostasis in primary human hepatocytes after 48 h.

Gene name*	log ₂ ratio	Fold change	p-Value	FDR
<i>Bile acid dependently up-regulated genes</i>				
FABP3	2.981	7.9	0.0001	0.009
APOL3	1.464	2.76	1.50E-10	1.09E-07
FGFR2	1.42	2.68	8.02E-06	0.001
LDLR	1.339	2.53	4.20E-13	6.58E-10
PPARD	1.256	2.39	2.18E-06	<0.001
FGF21	1.25	2.38	0.0281	0.347
FASN	1.185	2.27	0.0081	0.174
PCSK9	1.182	2.27	3.95E-06	0.001
S1PR2	1.146	2.21	0.0122	0.224
S1PR1	0.9341	1.91	2.49E-05	0.003
PRKCE	0.8938	1.86	0.0019	0.073
APOA2	0.8007	1.74	0.003	0.098
SCARB1	0.691	1.61	0.0017	0.068
CPT1A	0.6706	1.59	0.0018	0.071
SREBF2	0.6289	1.55	0.0071	0.162
LDLRAP1	0.6005	1.52	0.0061	0.149
NPC1	0.5843	1.5	0.0024	0.085
NPC1L1	0.5734	1.49	0.0014	0.061
SLC27A2	0.5539	1.47	0.0034	0.106
STARD3	0.5293	1.44	0.02643	0.336
FGF2	0.526	1.44	0.02203	0.305
PRKCA	0.5093	1.42	0.0053	0.137
FOXO3	0.5087	1.42	0.0284	0.349
AGPAT2	0.5037	1.42	0.0187	0.281
CREBBP	0.45	1.37	0.0355	0.391
FGFRL1	0.4191	1.34	0.0493	0.459
ATF6B	0.416	1.33	0.0352	0.389
<i>Bile acid dependently down-regulated genes</i>				
ACAT1	−0.432	0.74	0.02	0.291
PEX3	−0.4953	0.71	0.0115	0.216
AKR7A3	−0.5941	0.66	0.0041	0.118
APOM	−0.6031	0.66	0.0064	0.151
ACADSB	−0.6303	0.65	0.0008	0.042
APOC4	−0.7548	0.59	0.0086	0.182
AKR1C1	−0.8871	0.54	0.0161	0.261
APOH	−0.9548	0.52	0.0004	0.027
ANGPTL3	−0.9995	0.5	0.0009	0.045
ACAD11	−0.9997	0.5	2.95E-07	8.36E-05
APOA4	−1.059	0.48	0.0135	0.239
AKR1C4	−1.069	0.48	7.84E-08	2.66E-05
FABP7	−1.964	0.26	0.0051	0.134
HMGCS2	−2.128	0.23	0.0001	0.01

* Genes involved in lipid homeostasis (FDR < 0.46 and p-value < 0.05).

2.4. Processing of NGS data

RNA sequence reads were quality-checked using the software package fastqc (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) which computes various quality metrics for the raw reads. MessengerRNA sequencing reads were aligned to the genome and transcriptome using the R statistics package tophat v. 1.3.3 with default options. Before mapping, the low quality ends of the reads were clipped. The fragment length parameter was set to 100 bases with a standard deviation of 100 bases. Based on these alignments the distribution of the reads across genomic features was assessed. Isoform expression was quantified using the RSEM algorithm (R statistics package rsem) [23]. MicroRNA sequencing reads were aligned to the genome and quantified using ncPro-seq (<http://www.ncbi.nlm.nih.gov/pubmed/23044543>).

2.5. TaqMan analysis

Messenger RNA quantification was performed by transcribing 1.5 µg mRNA into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's recommendations. cDNA samples were diluted 1:5 and subsequently used in real-time PCR analyses (RT-PCR) by mixing 2 µl of cDNA and 8 µl of RT-PCR Universal Fast Master Mix specific to the respective cDNA target (TaqMan® Gene Expression Assays, Life-Technologies). β-actin was used to normalize measurements. miRNAs were quantified by transcribing 10 ng of extracted RNA into cDNA (TaqMan® miRNA Reverse Transcription Kit, Applied Biosystems, Rotkreuz, Switzerland) using stem-loop reverse transcription primers specific for the respective miRNA molecule (TaqMan® MicroRNA Assays, Life Technologies). RT-PCR analyses were performed using 0.67 µl of cDNA and 9.3 µl of the target-specific RT-PCR Universal Fast Master Mix (Applied Biosystems). All measurements were performed in triplicate.

2.6. Statistics

Generalized Linear Models (GLM) were used for statistical analysis comparing the mRNA and miRNA expression values across the different treatment conditions after NGS sequencing. One-sample t-tests were performed to compare the effects of CDCA on the expression of miRNAs versus DMSO as measured in TaqMan analysis. MiRNAs and mRNAs were correlated by Pearson's correlation analyses. Correlation diagrams were obtained using the "corrplot" library of the R-project, of mRNA and miRNA expression levels in both CDCA and DMSO treated cell lines, respectively. The statistical packages SPSS 22 and Graphpad Prism version 5 were used for statistical analyses. *p*-Values < 0.05 were considered significant.

3. Results

3.1. Effect of CDCA on genes involved in bile acid synthesis and transport, lipid, retinol and estradiol metabolism in hepatocytes

PHHs were treated with CDCA or DMSO (empty vehicle) for either 24 or 48 h to study the effect on the mRNA and miRNA expression profile. Changes in the expression of mRNAs were in general more pronounced after 48 h, and these results are therefore specifically discussed in the following section. Expression of 738 genes was significantly decreased and expression of 1566 genes was significantly increased (False Discovery Rate (FDR) in both cases ≤46%) after 48 h of CDCA treatment. When considering a FDR up to 30%, expression of 432 genes was significantly increased and expression of 1011 genes was significantly decreased in a BA-dependent manner. Gene enrichment analysis using the bioinformatics tool Metacore™ (Thomson Reuters) revealed that genes with altered expression in response to

CDCA belong primarily to gene networks involved in BA and lipid transport and metabolism, as well as estradiol and retinol metabolism (Fig. 1). As expected, CDCA increased expression of FXR-inducible genes such as *ABCB11* (BSEP), *FGF19*, *NROB2* (SHP) or *SLC51A* and *SLC51B* (OSTα, OSTβ) (Table 2).

Genes involved in the regulation of lipid homeostasis that showed altered expression in response to CDCA included several members of the apolipoprotein (*APO*) and fatty acid-binding protein (*FABP*) family, *SLC27A2* and *STARD3*, as well as genes involved in cholesterol, lipid and fatty acid (FA) synthesis and metabolism, such as *CPT1A*, *HMGCS2*, *FASN*, *ACAT1* and *PCSK9* (Table 3). Within signaling pathways influencing lipid homeostasis, *LDLR*, *PRKCA1*, *PPARδ*, *AGPAT2* and *SREBF2* were affected by CDCA. However, nuclear receptors known to control both BA and lipid homeostasis, e.g., FXR and LXR, were not significantly changed in their expression.

3.2. Effect of CDCA on the expression of drug-metabolizing enzymes

As shown in Table 2, CDCA modulated the expression of many genes involved in drug transport and phase I and II drug metabolism. Amongst drug-metabolizing cytochrome P450 enzymes, the expression of *CYP2E1*, *CYP1A1*, *CYP1A2*, *CYP2C8*, and *CYP3A4* was significantly decreased by CDCA. Amongst phase II enzymes, members of the UDP-glucuronosyl-transferase (UGT) and sulfotransferase (SULT) families in particular showed altered expression in response to CDCA. Specifically, expression of *UGT1A6*, *1A7* and *1A9*, *SULT1E1* and *SULT1B1* was decreased and expression of *UGT1A1*, *2A8* and *2B10*, and *SULT1C2* was increased by CDCA. Transcription factors that regulate drug metabolism and excretion, such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) or peroxisome proliferator-activated receptor γ (PPARγ) also showed altered expression in response to CDCA (Table 2).

Table 4

Effect of CDCA on the expression of miRNAs in primary human hepatocytes after 48 h.

miRNA*	log ₂ ratio	Fold change	<i>p</i> -Value	FDR
<i>Bile acid dependently up-regulated miRNAs</i>				
hsa-mir-552	1.588	3.006	0.0073	0.126
hsa-mir-1260a	1.243	2.367	<0.0001	0.002
hsa-mir-149	1.207	2.309	0.0052	0.115
hsa-mir-1260b	1.186	2.275	<0.0001	0.004
hsa-mir-3651	1.129	2.187	0.0032	0.115
hsa-mir-4485	0.9179	1.889	0.0038	0.115
hsa-mir-3607	0.8542	1.808	0.0043	0.115
hsa-mir-505	0.8398	1.79	0.0035	0.115
hsa-mir-6723	0.7282	1.657	0.0052	0.115
hsa-mir-15b	0.7139	1.64	0.0036	0.115
hsa-mir-328	0.7	1.625	0.0081	0.133
hsa-mir-92a-2	0.6913	1.615	0.0091	0.133
hsa-mir-204	0.5362	1.45	0.0092	0.133
hsa-mir-885	0.4937	1.408	0.0097	0.133
<i>Bile acid dependently down-regulated miRNAs</i>				
hsa-mir-34a	−0.44	0.737	0.0087	0.133
hsa-mir-30a	−0.5096	0.702	0.0055	0.116
hsa-mir-98	−0.6309	0.646	0.0062	0.122
hsa-mir-5590	−0.8854	0.541	0.0036	0.115
hsa-mir-2355	−0.9401	0.521	<0.0001	0.004
hsa-mir-190a	−1.1	0.467	0.0022	0.115
hsa-mir-190b	−1.266	0.416	0.0096	0.133
hsa-mir-2467	−1.421	0.373	0.0038	0.115
hsa-mir-2114	−1.653	0.318	0.005	0.115
hsa-mir-452	−1.847	0.278	<0.0001	0.002
hsa-mir-486	−2.285	0.205	0.0047	0.115
hsa-mir-486-2	−2.287	0.205	0.0048	0.115
hsa-mir-451b	−2.461	0.182	0.0069	0.125
hsa-mir-451a	−2.461	0.182	0.0069	0.125
hsa-mir-6503	−2.9	0.134	0.0009	0.073

* Shown are all CDCA dependently modulated microRNAs with a FDR ≤ 0.133 and a *p*-value < 0.01.

3.3. The impact of CDCA on the miRNA expression profile in primary human hepatocytes

Whereas only few changes in the miRNA profile were observed after 24 h' treatment of PHHs with CDCA, expression of 52 miRNA molecules was significantly increased and that of 29 miRNAs was significantly decreased after CDCA treatment for 48 h, as shown in Table 4 (FDR < 0.133, $p < 0.01$). Results were confirmed by TaqMan analysis remeasuring and confirming the CDCA dependent downregulation of 3 microRNAs (miR-6503, miR-486 and miR-223) in three separate hepatocyte batches. These microRNAs belong to the group of miRNA molecules most strongly affected by CDCA, thus, allowing a confirmatory trend determination in expression by TaqMan analyses. Other microRNAs affected by CDCA included miR-552, miR-149, and miR-886 (increased) and miR-34a, miR-30a, miR-452, miR-486 and miR-190a and 190b (decreased). Figs. 2 and 3 illustrate the CDCA-dependent miRNA profile as a volcano plot and heatmap, respectively. The heatmap integrated cluster analysis shows considerable interindividual variability in the miRNA expression profile following CDCA treatment.

3.4. Correlation of CDCA-induced changes in miRNA levels with mRNA expression

To evaluate the extent to which miRNAs modulated by CDCA could influence the expression of genes involved in BA synthesis, transport and metabolism, Pearson's correlation analyses were performed. Only those miRNAs (shown in Table 4) that were predicted to bind to at least three mRNAs contained in the gene cluster regulating BA

homeostasis (Table 2), lipid metabolism (Table 3) and drug metabolism (Table 2) were included in the correlation analyses. Binding of miRNAs was predicted using the bioinformatics tool mirDIP (<http://ophid.utoronto.ca/mirDIP>) [24]. As shown in Fig. 4 a distinct cluster of mRNAs consisting of *ABCG5* and *ABCG8*, *SLC22A7*, *NR0B2*, *SLC51B*, *SLC10A1*, *CYP3A4* and *FGF19* is inversely associated with the expression of miR-34a upon CDCA treatment. The same gene cluster appears to be strongly positively correlated with miRNAs –885, –15b and –505, leading to the hypothesis that miRNA and mRNA clusters may be regulated by common transcriptional pathways. As demonstrated in Fig. 5, especially microRNA-1260a shows strong inverse correlations with several important genes involved in lipid homeostasis, including *AGPAT2*, *S1PR2*, *CPT1A*, *APOM*, *AKR1C1* and *LDLR* upon CDCA treatment pointing to an inhibitory effect of miR-1260a on the expression of this gene battery. In contrast to miR-1260a, miRNA-98 that is strongly inversely correlated with a gene battery including amongst others *NPC1L1*, *HMGCS2*, *AGPAT2*, *FASN*, *STARD3* and *S1PR2*, loses many of those inverse associations upon CDCA treatment. This observation points to a less strong regulatory impact of miR-98 on the lipid gene network upon higher hepatic BA concentrations. Fig. 6 demonstrates the correlation behavior of mRNAs belonging to the drug metabolism network and distinct microRNAs. Interestingly miRNA-1260 comes here to the fore again, now with two family members -1260a and -1260b, showing both strong positive correlations with the drug metabolizing enzymes *SULT1E1*, *CYP1A1* and *CYP1A2* and *CYP2E1*. >Less strong but still relevant positive associations in gene expression are also observed with the UGT family members *UGT2A3*, *UGT1A7*, *UGT1A8* and *CYP2C8*. This observation suggests a connection between miR-1260 expression and the

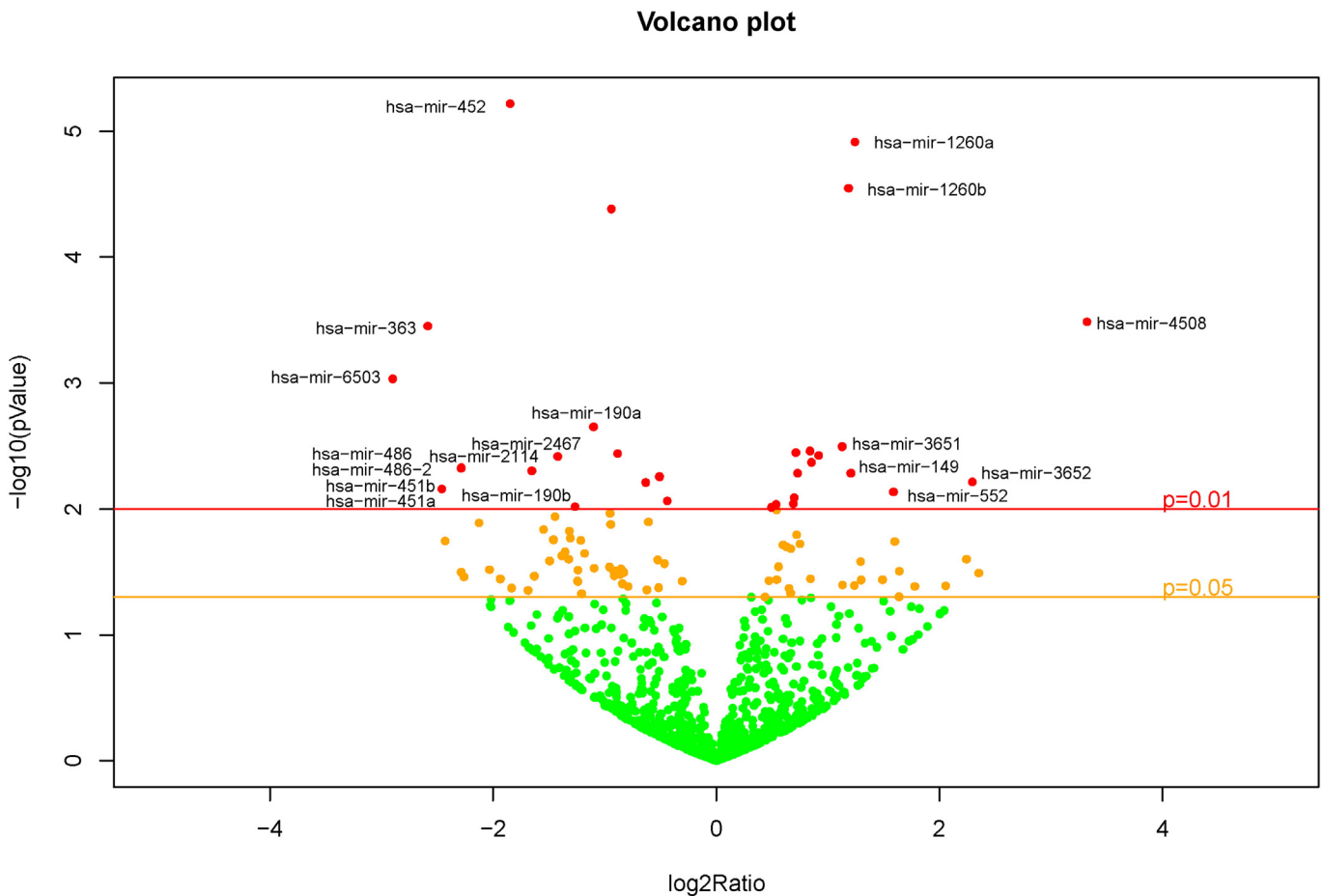


Fig. 2. Effect of CDCA on the global miRNA profile in five batches of primary human hepatocytes. Dots represent the average fold-change of miRNAs shown as \log_2 values (x-axis) and corresponding p -values represented as $-\log_{10}$ values (y-axis) when comparing CDCA and empty vehicle (DMSO) treated cells. Red: miRNAs with p -values < 0.01. Yellow: miRNAs with p -values < 0.05. Green: miRNAs with p -values > 0.05. Labeled miRNAs represent molecules that showed a ≥ 2 fold increase or decrease.

suggests a key role of miR-34a in the autoregulation of bile acid homeostasis. We observed a strong downregulation of miR-34a, which is well in line with results published by Castro et al. (2013), showing a suppressive effect of ursodeoxycholic acid in patients suffering from NAFLD [25]. The suppressive effect of CDCA on miR-34a expression suggests also consequences for NAFLD development, and the regulation of energy homeostasis and cancer related pathways. MicroRNA-34a has been shown to SIRT-dependently regulate brown fat formation. The observed upregulation of serum miR-34a in NAFLD patients underlines the importance of miR-34a in metabolic diseases [26]. MiRNA-34a has been studied in the context of cancer development and tumor growth. Associations with disease development, prognosis and severity have been described for bladder, breast, liver and colon cancer as well as lymphoma [27–30]. Besides miR-34a, we detected a distinct microRNA cluster, composed of miR-885, miR-505 and miR-15b, to be CDCA dependently modulated in expression. These microRNAs were significantly upregulated and showed, in contrast to miR-34a – a positive correlation with the group of genes inversely associated with the expression of miR-34a. It is conceivable that these three miRNAs and the associated BA gene cluster are co-regulated by a common transcriptional pathway that is activated by CDCA. MiRNA-885 has been shown to play a role in the pathogenesis of different cancer types [31,32] and has also been discussed as a potential serum marker for the detection of ongoing liver pathologies [33]. MiR-15b has been shown to be up-

CDCA had both enhancing and suppressing effects on the expression of miRNAs, with the majority of the miRNA molecules having decreased levels under CDCA treatment. Importantly, we show that CDCA modulates the expression of distinct miRNAs that appear to be connected to the expression of gene clusters within the bile acid, lipid and drug homeostasis associated gene networks. MicroRNA-34a is CDCA dependently and strongly inversely correlated with key genes involved in BA homeostasis including FGF19, NR0B2 (SHP), OST α / β , ABCG5/ABCG8, and SLC22A7. This finding

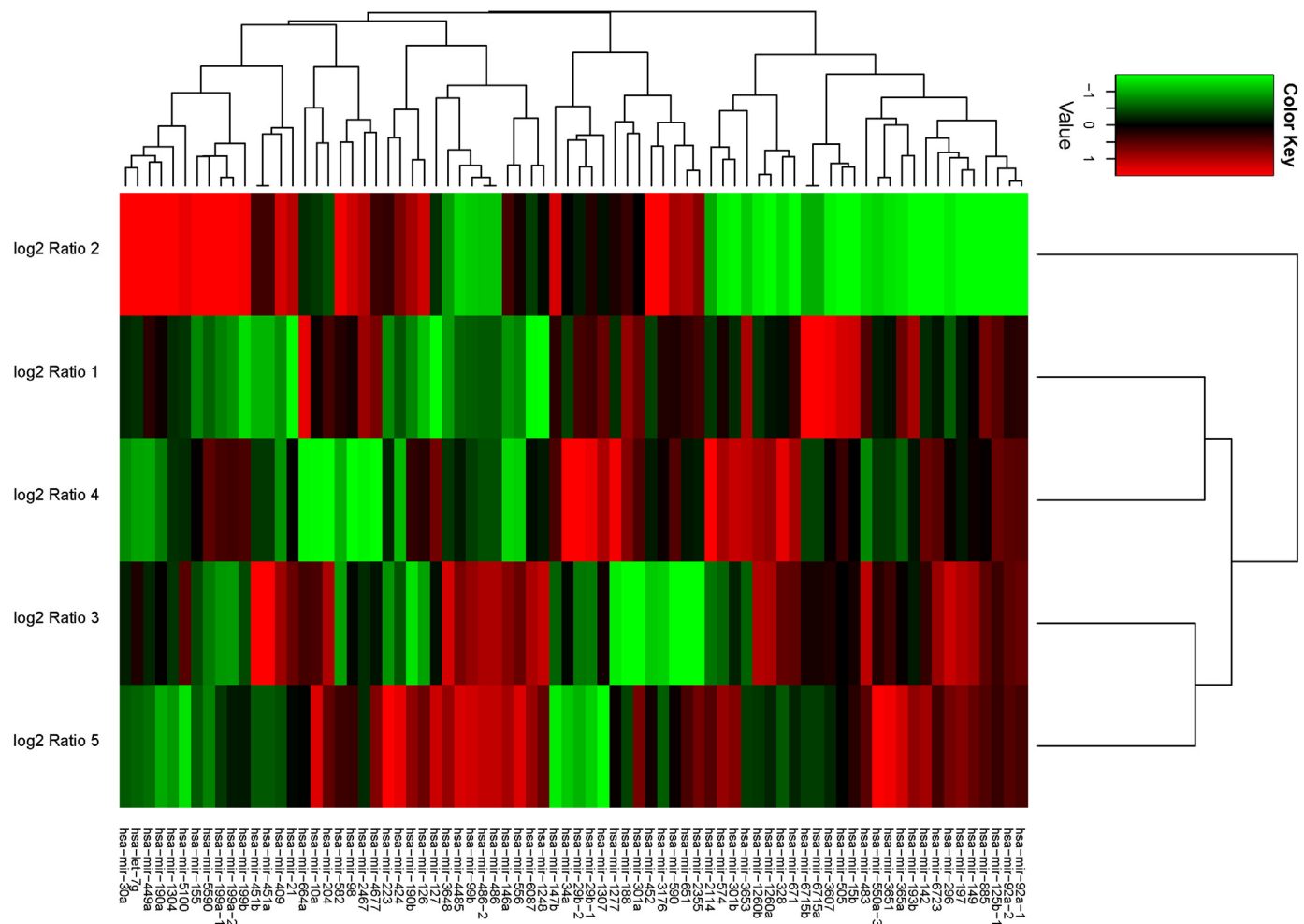


Fig. 3. Heat map showing CDCA-induced changes in miRNA profile clustering in five batches of primary human hepatocytes. MiRNAs with changes of at least 50% and *p*-values <0.05 are shown. Red: increased miRNAs. Green: decreased miRNAs.

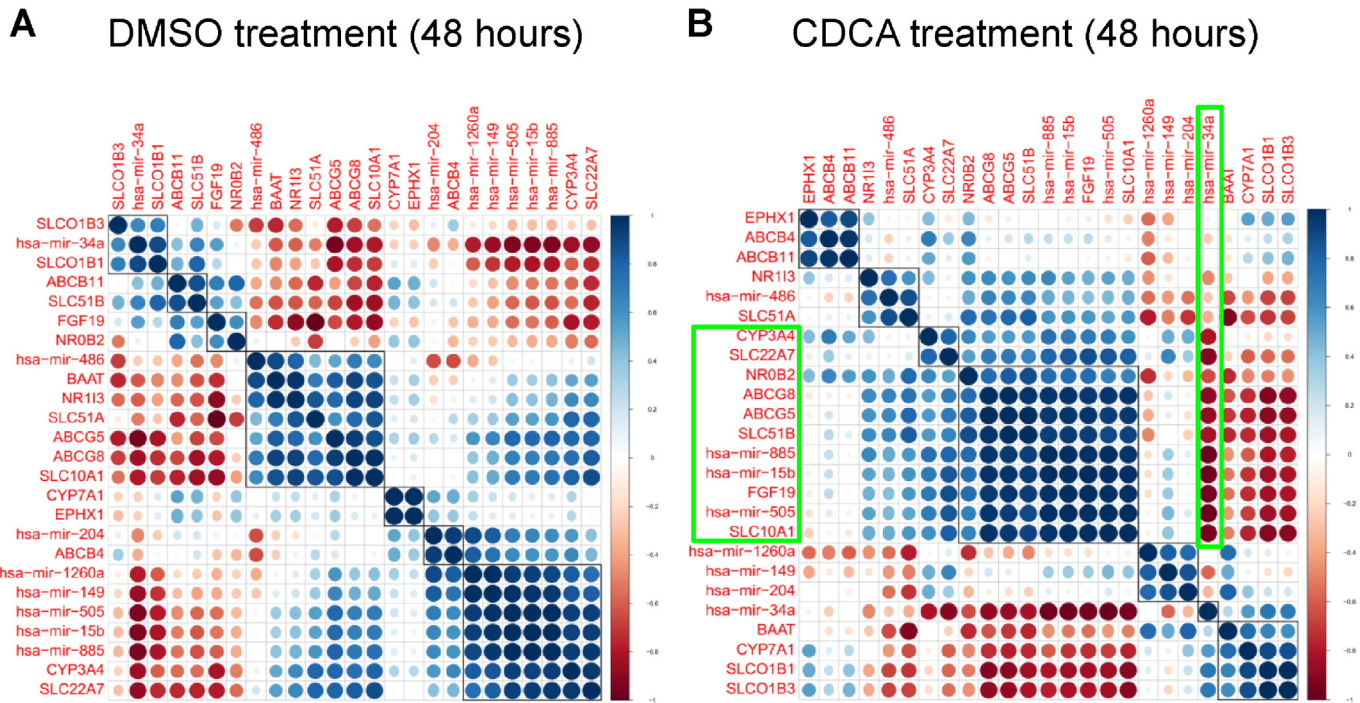
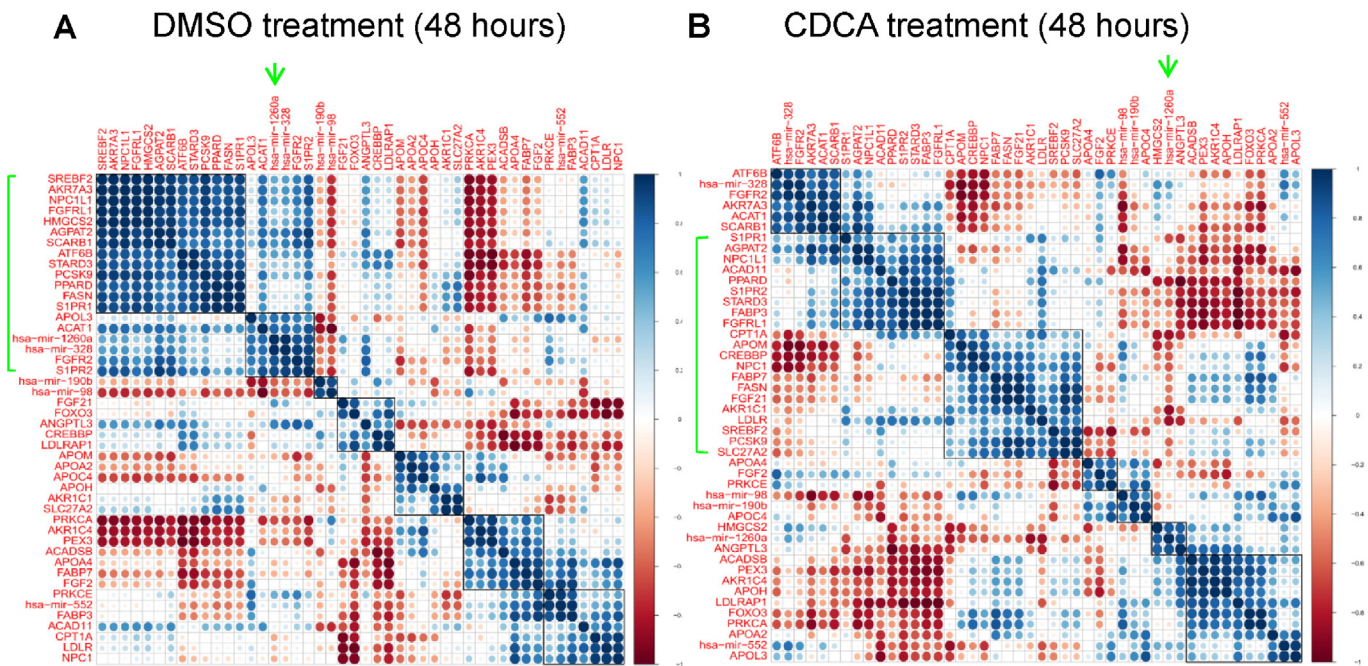


Fig. 4. Pearson's correlation analyses on mRNA and miRNA expression levels taking genes from the BA gene cluster into consideration. DMSO treatment (A), CDCA treatment (B). MicroRNAs with predicted binding sites in at least three target genes within the bile acid (BA) gene cluster and significantly up- analyses on mRNA and miRNA expression levels taking genes from the BA gene cluster into consideration. DMSO treatment (A), CDCA treatment (B). MicroRNAs with predicted binding sites in at least three target genes within the bile acid (BA) gene cluster and significantly up- or downregulated (FDR ≤ 0.133) were included. Shown are BA mRNA targets that were significantly up or downregulated by CDCA (FDR < 0.46). Blue, positive correlation; red, inverse correlation. A distinct gene cluster composed of important BA homeostasis regulating genes, comprising *SLC51A*, *CYP3A4*, *SLC22A7*, *NR0B2*, *ABCG5/8*, *SLC51B*, *FGF19* and *SLC10A1* appears to be strongly inversely correlated with miRNA-34a and positively correlated with the microRNAs miR-885, miR-15b, and miR-505 upon CDCA treatment (highlighted in green).

regulated in NAFLD, and thus, has been suggested to play a role in the pathogenesis of this disease [34]. MicroRNA-505-3p is discussed as putative biomarker for primary biliary cirrhosis [35].

CDCA strongly affected genes involved in lipid homeostasis, including *FGF2*, *FGFR2*, *HMGCS2*, *FABP* and *APO* family members. As potent ligands for FXR, CDCA and other BAs regulate genes involved in



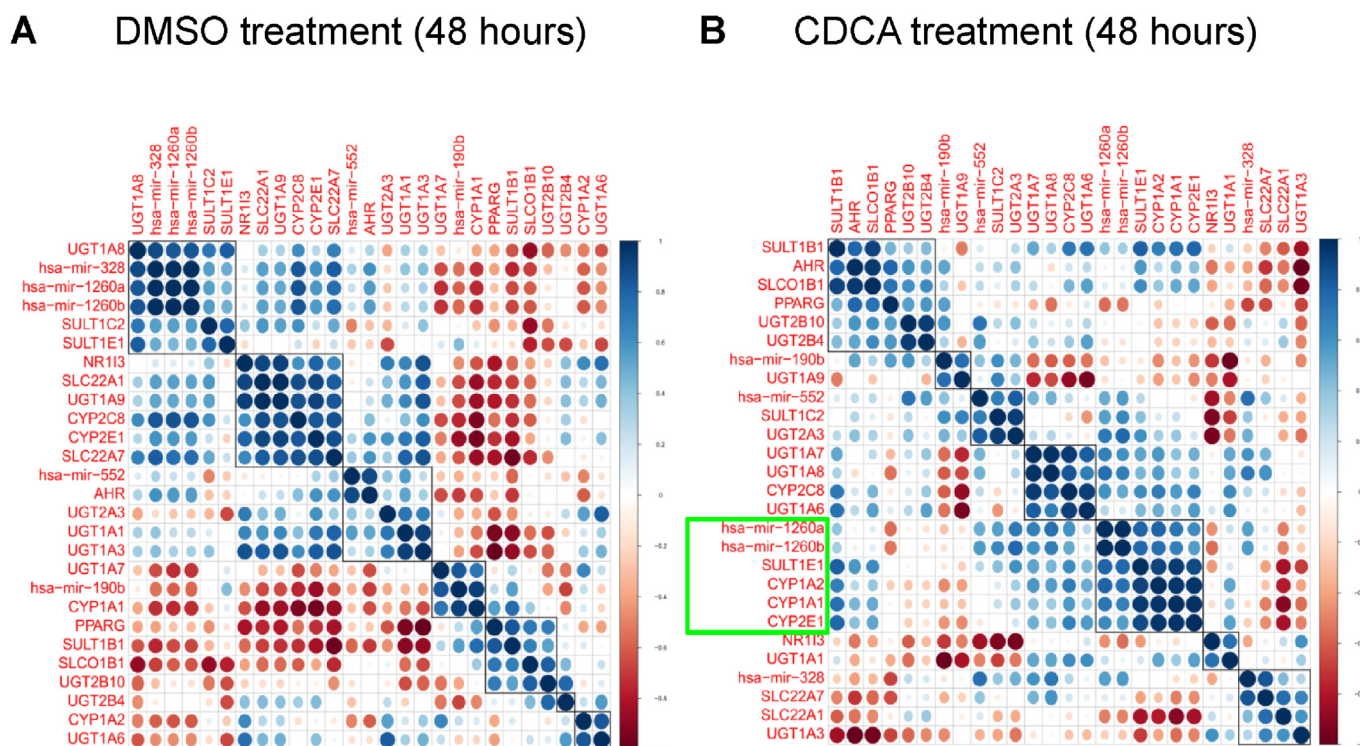


Fig. 6. Pearson's correlation analyses on mRNA and miRNA expression levels taking genes from the DM gene cluster into consideration. DMSO treatment (A), CDCA treatment (B). Analyses were performed including those miRNAs that were significantly up- or downregulated and for which binding sites in at least three target genes within the drug transport and metabolism (DM) gene cluster were predicted. Shown are DM mRNA targets that were significantly up- or downregulated. Blue, positive correlation; red, inverse correlation. A distinct gene battery, composed of important genes encoding drug metabolizing enzymes, including SULT1E1, CYP1A2, CYP1A1 and CYP2E1 and to a less extent several UGT family members and CYP2C8, is positively correlated with the expression of miR-1260a and miR-1260b upon CDCA treatment (highlighted in green).

cholesterol, FA, and lipid metabolism, either by direct transcriptional activation or by FGF-dependent hormonal signaling. In vivo studies performed in mice have shown that orally administered BAs lead to a positive effect on body weight [36] and lower the risk for NAFLD under high-fat diet conditions [2]. Our results support the notion that BAs modulate the expression pattern of genes involved in lipid synthesis, transport and metabolism, which could contribute to the lipid lowering properties repeatedly observed with BAs. In this context we would like to highlight the enhancement of miR-1260 expression upon CDCA treatment, which was associated with a strong parallel expression of genes involved in xenobiotics metabolism (CYP2E1, CYP1A1, CYP1A2) and an inverse expression of important genes involved in lipid homeostasis (i.e. CPT1A, APOM, LDLR). This finding points to a putative coregulatory element regulating the expression of miR-1260 and the mentioned drug gene group and a role of this microRNA as regulatory element in lipid metabolism. MicroRNA-1260 has been recently described as putative biomarker for paclitaxel-induced apoptosis in HCC cells [37]. Our systematic analysis of genes involved in drug metabolism and transport show, that many genes belonging to phase I (CYP1A members, CYP2E1, CYP2C8), phase II (several genes of the UGT and SULT family) and phase III (SLC22A1, SLC22A7) are significantly modulated by CDCA. This observation allows the speculation that bile acid derivatives may have the potential to induce drug-drug interactions with other compounds through the modulation of DM gene expression. The importance of our findings with regard to a relevant interplay of BAs and DMEs is further underlined by the fact that distinct and relevant drug-drug interactions with BAs have been earlier described in other studies. This comprises amongst others the interaction between the immunosuppressant Cyclosporine A and bile acids. Cyclosporine A has been demonstrated to lead to a significant accumulation of bile acids and the development of a cholestatic feature

in liver derived HepaRG cells [38]. Another important example comprises the interaction of the calcium channel blocker nitrendipine and CDCA. Sasaki and co-workers demonstrated that CDCA and UDCA are able to inhibit nitrendipine absorption by 50% and thus to decrease plasma concentrations of nitrendipine to a clinically relevant extent in healthy individuals [39].

Because the five batches of PHHs were obtained from patients resected for liver metastases that were incurred through different malignant tumors such as colon or kidney cancer, it cannot be excluded that the underlying pathologies or the existence of genetic polymorphisms within the discussed gene clusters or in genes coding for key transcription factors could induce an additional variance in gene expression. We specifically concentrated on the question to what extent CDCA is able to change the microRNA profile, as also optimally measurable in the chosen time frame of 48 h. It would be interesting to further study in future studies how and to what extent other epigenetic regulatory mechanisms, such as e.g. methylation or histone acetylation, are influenced by CDCA. Correlation analyses were done at the miRNA/mRNA level. Because the regulatory effect of miRNAs is often only observed at the protein level, we cannot exclude the possibility that the miRNAs have additional effects on targets that were not detected by assessment of mRNA levels alone.

We conclude that the expression of the human miRNAome and mRNAome in PHHs are significantly modulated by the bile acid CDCA, with relevant consequences for the functionality of gene networks involved in bile acid, lipid and drug metabolism. CDCA-induced regulation by miRNAs may exert downstream effects on genes within functionally important networks. Our findings give important novel insights into the ability of BA derivatives to induce relevant changes in gene networks relevant for BA compound safety and metabolic disease development such as obesity and NALFD.

Conflicts of interest

None of the authors declares any conflicts.

Author contributions

RK, GK and JM contributed to study concept and design and data acquisition; RK, SML, WT and JM contributed to data acquisition; RK, AB, GK, and JM contributed to analysis of the data; RK, AB, GK, HBS and JM contributed to writing the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.lfs.2016.04.037>.

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III. CHAPTER 2

microRNA-192 suppresses the expression of the farnesoid X receptor

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microRNA-192 suppresses the expression of the farnesoid X receptor

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Krattinger R, Boström A, Schiöth HB, Thasler WE, Mwinyi J, Kullak-Ublick GA. microRNA-192 suppresses the expression of the farnesoid X receptor. *Am J Physiol Gastrointest Liver Physiol* 310: G1044–G1051, 2016. First published April 14, 2016; doi:10.1152/ajpgi.00297.2015.—Farnesoid X receptor (FXR, *NR1H4*) plays an important role in the regulation of bile acid homeostasis in liver and intestine and may exert protective effects against certain forms of cancer such as colon carcinoma. However, the role of FXR in cell growth regulation, apoptosis, and carcinogenesis is still controversial. Similar to FXR, microRNA-192 (miR-192) is mainly expressed in the liver and colon and plays an important role in the pathogenesis of colon carcinoma. In this study, we investigated the extent to which FXR is regulated by miR-192. Two *in silico*-predicted binding sites for miR-192-3p within the *NR1H4*-3' untranslated region (UTR) were examined *in vitro* by luciferase reporter assays. Wild-type and mutated forms of the *NR1H4*-3' UTR were subcloned into a pmirGLO vector and cotransfected into Huh-7 cells with miR-192-3p. To study the effects of miR-192 on the expression of FXR, FXR target genes and cell proliferation, Huh-7 and Caco-2 cells were transfected with miR-192-5p and -3p mimics or antagomirs. In addition, the correlation between FXR and miR-192 expression was studied by linear regression analyses in colonic adenocarcinoma tissue from 27 patients. MiR-192-3p bound specifically to the *NR1H4*-3' UTR and significantly decreased luciferase activity. Transfection with miR-192 led to significant decreases in *NR1H4* mRNA and protein levels as well as the mRNA levels of the FXR-inducible bile acid transporters OST α -OST β and OATP1B3. Significant inverse correlations were detected in colonic adenocarcinoma between *NR1H4* mRNA and miR-192-3p expression. In summary, microRNA-192 suppresses the expression of FXR and FXR target genes *in vitro* and *in vivo*.

miR-192; farnesoid X receptor; bile-acid transporters; drug-induced liver injury; colonic adenocarcinoma

NUCLEAR FARNESOID X RECEPTOR (FXR, *NR1H4*) is a ligand-activated transcription factor that plays a crucial role in the regulation of bile acid, cholesterol, lipid, and glucose homeostasis. It is mainly expressed in the liver, intestines, kidney, and adrenal glands (34). FXR regulates key genes involved in human bile acid synthesis and metabolism, including bile acid transporters (4). Studies on FXR knockout mice have shown that FXR exerts hepatoprotective effects. Diminished FXR expression has been linked to an increase in inflammatory responses and neoplastic transformation in mice (7, 16). Mice lacking FXR expression show elevations in serum and hepatic bile acid levels and a higher incidence of hepato-

(cholangio)cellular carcinoma (12, 33). In mouse intestine, loss of FXR and subsequent elevations of intestinal bile acid concentrations lead to earlier mortality caused by increased tumor progression via promotion of Wnt signaling. FXR may play a key role in the intestinal defense against potentially toxic bile acids by regulating their transport, detoxification, and neosynthesis (8, 23). Decreased FXR expression levels in human colon cancer tissue compared with nonneoplastic tissue are associated with adverse clinical outcome (15). In contrast, strongly enhanced FXR expression, leading to altered expression of FXR-regulated drug uptake transporters, confers chemoresistance in cancer patients (22). The role of FXR in cell growth regulation, apoptosis, and carcinogenesis is controversially discussed in the literature. An immunohistochemical study, for example, showed preserved or enhanced FXR protein expression in tumor cell nuclei of human hepatocellular carcinoma tissue compared with hepatocyte nuclei of normal and diseased liver (14).

MicroRNAs (miRs) are short noncoding RNA molecules of 18–25 nucleotides in length that repress specific target mRNAs by degradation or translational repression (2). Two important liver-specific miRNAs are miR-122, which is estimated to comprise 70% of the total hepatic miR pool in adults, and miR-192 (28). The gene encoding hsa-miR-192 is located on chromosome 11. In addition to expression in the liver, miR-192 is found in the kidneys and gastrointestinal tract (20). Elevated serum levels of miR-192 have been detected in various liver-associated diseases, including drug-induced liver injury, non-alcoholic steatohepatitis, cholangiocarcinoma, and hepatitis B-related hepatocellular carcinoma and may serve as a biomarker (24, 26, 28, 31, 37). Thus several studies have hypothesized that tissue-specific chronic inflammation may trigger increases in miR-192 expression, as in nonalcoholic steatohepatitis (21, 24, 26). Because miR-192 shows an inverse correlation with the metastatic potential of colon cancer cells, miR-192 has been suggested to be a predictive biomarker for the risk of developing liver metastasis in colon carcinoma. *In vivo* studies in mice suggest that miR-192 targets B-cell lymphoma 2 (*BCL2*), zinc-finger E-box-binding homeobox 2 (*ZEB2*), and vascular endothelial growth factor A (*VEGFA*), all of which are important antiapoptotic and angiogenic regulators (9). The tumor suppressor protein p53 can act as a transcription factor within the miR-192 promoter, whereas miR-192 itself appears to suppress carcinogenesis by promoting p21 accumulation (3, 27). Loss of p53 functions by mutation and consequently decreased expression of miR-192 has been suggested to be a key step in colon carcinogenesis (3, 11, 25, 29). Although miR-192 is not considered to be a typically dysregulated microRNA in several studies on hepatocellular carcinoma

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(HCC), Lian et al. (19) showed a significantly suppressed expression of miR-192 in HCC tissue compared with nontumorous tissue. An important role of miR-192 targeting *ZEB2* mRNA has also been found in HCC (13). In contrast, certain forms of cancer, such as cholangiocarcinoma and esophageal cancer, show increased miR-192 expression during carcinogenesis (21, 26).

In this study, we investigated to what extent miR-192 modulates the expression of FXR and thereby affects the expression of FXR target genes in liver and colon cancer-derived cell lines.

MATERIALS AND METHODS

Bioinformatics. An in silico search for possible miRNA-binding sites in the 3' untranslated region (UTR) of the *NR1H4* gene was performed by using miRANDA (Memorial Sloan-Kettering Cancer Center, New York, NY), DIANA-microT-CDS (Biomedical Science Research Center Alexander Fleming, Athens, Greece) and miRBase (Faculty of Life Science, University of Manchester, Manchester, UK). mRNA and miR expression data from 27 colonic adenocarcinoma tissue samples [E-GEOD-29623, Affymetrix GeneChip Humane Genome U133 Plus 2.0 and NIH TaqMan microRNA Array v.2 (5)] were retrieved from the openly accessible platform ArrayExpress (EMBL-European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, UK).

Cell culture. The human hepatoma-derived Huh-7 and colon carcinoma-derived Caco-2 cell lines (American Type Culture Collection, Molshheim, France) were cultured in RPMI-1640 and Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA), respectively, supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA). Primary human hepatocytes (PHHs) obtained from three patients suffering from primary or secondary liver carcinoma (ethical approval by the Local Ethical committee of the University of Munich, Germany, and the Ethics Committee of the Canton of Zurich, Switzerland) were isolated from the cancer-adjacent normal tissue and cultured as described (17). PHHs were kept in maintenance medium including ultraglutamine for 5 h before further procedures. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Transient transfection with miR-192. To investigate the effect of miR-192 on *NR1H4* mRNA and protein expression, Huh-7 and Caco-2 cells were seeded in 12-well plates (8×10⁴ and 4×10⁵ cells/ml, respectively). After 24 h, cells were transfected with 100 nM hsa-miR-VANA miRNA mimics or anti-miR miRNA inhibitors (hsa-miR-192-3p/-5p and corresponding negative controls, Life Technologies) by using Lipofectamine RNAiMax diluted in Opti-MEM I (both purchased from Invitrogen) at a final concentration of 3 mM. Hsa-miR-1 and its known suppressive effect on twinstin-1 (TWF-1) expression were used as a positive control. After 4 h of incubation, the transfection medium was replaced with fresh complete growth medium. At 24, 48, and 72 h after transfection, total mRNA and protein were isolated by use of TRIzol reagent (Life Technologies) and RIPA buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate (all from Sigma-Aldrich), 1 mM EDTA (Pharmacia Biotech, Uppsala, Sweden), and 0.1% SDS and 10% glycerol (both from Sigma-Aldrich)], respectively. FXR mRNA and protein were quantified in three independently performed experiments.

To examine miR-192-dependent expression of FXR target genes, Huh-7 cells were treated with 50 µM chenodeoxycholic acid (CDCA) (Sigma-Aldrich) for 24 h. Data obtained were merged from four experiments, and the three most representative values were averaged. To prove that miR-192 mimics control the expression of FXR target genes through FXR regulation, 100 nM FXR small interfering RNA

(siRNA) or corresponding negative control (Life Technologies) were transfected simultaneously with the miRNA mimics for 48 h.

Reverse transcription and quantitative real-time PCR. Extracted RNA (1.5 µg) was transcribed into cDNA by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) according to the manufacturer's protocol. A total of 2 µl of 1:5 diluted cDNA was mixed with 8 µl RT-PCR Universal Fast Master Mix (Applied Biosystems) including specific primers (TaqMan Gene Expression Assays *NR1H4*, *SLCO1B3*, *SLC51A*, and *SLC51B*; Life Technologies) and subjected to real-time PCR. β-Actin was used as an internal control. To measure miR expression, 10 ng of extracted RNA was reverse transcribed into cDNA by using a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) and specific stem-loop reverse transcription primers (TaqMan MicroRNA Assays hsa-miR-192-3p and hsa-miR-192-5p; Life Technologies). RT-PCR was performed by using 0.67 µl cDNA and 9.3 µl RT-PCR Universal Fast Master Mix (Applied Biosystems) including primers. U6snRNA was used as an internal control. Mean miR expression in three passages of cells were analyzed per cell line. All measurements were performed in triplicate.

Western blot analysis. Protein samples (17–30 µg) were diluted 1:5 with loading buffer, denatured, and separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membranes and preincubated for 1 h in blocking buffer and then for 16 h with the primary antibodies (anti-FXR, Santa Cruz Biotechnology, Dallas, TX; anti-β-actin, Abcam, Cambridge, UK) at 4°C. After four washing steps, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit, Thermo Scientific, Waltham, MA) for 1 h at room temperature. Protein bands were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany). β-Actin was used as a housekeeping gene.

Transient cotransfection with miR-192-3p (miR-192*) and NR1H4 3' UTR plasmid constructs and luciferase reporter assays. To examine the *NR1H4* 3'UTR as a target of miR-192-3p in vitro, luciferase assays were performed with a pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Fitchburg, WI), containing both the coding sequences of firefly luciferase and *Renilla* luciferase (internal control). The *NR1H4* 3'UTR (accession number NG_029843.1) was cloned into the pmirGLO vector system by using specific primers (Table 1). Plasmids were verified by Sanger sequencing. Huh-7 cells were seeded in 48-well plates (1.6×10⁵ cells/ml). After 24 h, the cells were transfected with 50 nM hsa-miR-VANA miRNA mimics or a negative control (hsa-miR-192-3p, Negative Control no. 1; Life Technologies) and 100 ng/well of plasmid DNA with use of Lipofectamine 2000 (Invitrogen). The activities of firefly and *Renilla* luciferases were measured at 24 h after cotransfection using the Dual-Luciferase Reporter 1000 Assay System (Promega) according to the manufacturer's protocol. Hsa-miR-21-5p and its effect on the miR-21 target sequence were used as a positive control. Analysis was performed using the GloMax Multi Detection System (Promega). An empty pmirGLO vector was used as a background control. QuikChange Multi Site-Directed Mutagenesis and QuikChange II XL Site-Directed Mutagenesis Kits (Agilent Technologies, Santa Clara, CA) were used to introduce mutations into the miR-192-3p-binding sites using specific primers (Table 1). Three independent luciferase reporter assays were performed including wild-type and mutated target sequences.

Cell proliferation and invasion assays. Cell proliferation was determined by using the alamarBlue (Thermo Scientific) cell viability reagent according to the manufacturer's instructions. Briefly, Huh-7 and Caco-2 cells were seeded in 96-well plates (7.5×10⁴ and 2×10⁵ cells/ml, respectively). After 24 h, cells were transfected with 100 nM miR-192 mimics, FXR siRNA, or the corresponding negative controls. After 48, 72, and 96 h 10 µl of alamarBlue was added to each well and cells were incubated for 4 h at 37°C. The absorbance was

Table 1. Primer sequences used for subcloning and mutagenesis

Oligonucleotide	Sequence (5'–3')	Purpose
FXR_3UTR_FW	<u>aggagctct</u> ggggattacaggggagg	Subcloning of <i>NR1H4</i> 3' end into pmirGlo
FXR_3UTR_RV	<u>aggtcgacg</u> ccaagattgaatacaactct	Subcloning of <i>NR1H4</i> 3' end into pmirGlo
FXRmut_miR192*_FW1	ggaatcctgcattctc <u>gtcgcgcg</u> agccctgtttgcctaattaaattg	Multisite-directed mutagenesis
FXRmut_miR192*_FW2	gagttgtattcaatct <u>ggccg</u> tcgacctaatcccgcggc	Multisite-directed mutagenesis
FXRwt_miR192*_FW	gagttgtattcaatct <u>ttggcag</u> tcgacctaatcccgcggc	Site-directed mutagenesis
FXRwt_miR192*_RV	gccgcgggattaggtcgac <u>tgc</u> ccaagattgaatacaactc	Site-directed mutagenesis

Recognition sites for the restriction enzymes are underlined in the primers used for subcloning. In silico-predicted binding sites for hsa-miR-192-3p are underlined in the mutagenesis primers, whereas mutations inserted into these sites are indicated in bold. FXRwt_miR192* primers were used to optimize the second binding site for miR-192-3p.

determined at 560/600 nm by using the GloMax Multi Detection System.

Cell invasion was determined with extracellular matrix-coated invasion chambers (QCM 24-well cell invasion assay, Millipore, Billerica, MA) according to the manufacturer's instructions. Huh-7 and Caco-2 cells were harvested and resuspended in serum-free medium after pretreatment for 48 h with miR-192 mimics or negative control (cell density for transfection: 1×10^5 and 4×10^5 cells/ml, respectively). Then, 1×10^5 cells were plated into the invasion chamber, whereas the bottom well of the chamber contained 500 μ l of the corresponding medium supplemented with 10% FBS. After 48 h of incubation, the invaded cells on the underside of the membrane were detached, lysed, and stained with CyQuant GR Dye (Millipore). Fluorescence was measured by use of a 490/510–570 filter set and GloMax Multi Detection System.

Statistical analysis. Paired one-sample *t*-tests were performed to compare the effects of miR-192 mimics, antagomirs, FXR siRNA and the corresponding negative controls on FXR mRNA/protein expression levels and on cell proliferation/invasion. Luciferase activities were compared between miR-192-3p- and mock-transfected cells by one-way analysis of variance. All data obtained from transfection experiments were compared with negative control mimics or inhibitors, in which expression levels in mock-transfected cells were defined as 1 (except for cell proliferation assays, where values were normalized to the 48-h time point of a particular condition). In general, the negative controls were not expected to bind to the *NR1H4* 3'UTR or to block the activity of endogenously expressed miR-192. Only experiments with verified positive controls were included in statistical analyses. The association between FXR and miR-192 expression levels was investigated in 27 colonic adenocarcinoma tissue samples by linear regression analyses excluding subjects that had undergone chemotherapy [E-GEOD-29623 (5)]. Values are shown as averages \pm standard deviation. A *P* value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed with R Software (version 2.15.2) and GraphPad Prism (version 5.04).

RESULTS

The mature forms of pre-miR-192 derived from the 5' and 3' strands of the precursor are detectable in Huh-7 and Caco-2 cells. The online database miRBase lists two mature sequences for the human miRNA precursor pre-miR-192, miR-192-5p, and miR-192-3p. Quantitative measurement of the endogenous expression levels showed the presence of both strands at considerable amounts in Huh-7, PHH, and Caco-2 cells. MiR-#3p was expressed 10-fold less in Huh-7 cells, 32-fold less in Caco-2 cells, and 39-fold less in PHH cells compared with miR-#5p. Furthermore, miR-192-3p showed a 9- to 10-fold

higher expression in Caco-2 cells than in the hepatoma cell line or the primary human hepatocytes (data not shown).

Confirmation of the *NR1H4* 3'UTR as a target of miR-192-3p by luciferase reporter assays. The software tools miRANDA and DIANA-microT-CDS predicted binding of miR-192-3p to *NR1H4* transcript positions 199–227 and 324–

A

	3' gacacuggauaccUUAACCGUc	5' hsa-miR-192-3p
207:	5' aaucugcauucuAAUUGGCAa	3' <i>NR1H4</i> 3' prime
	3' gacacuggauaccuuAACCGUc	5' hsa-miR-192-3p
332:	5' gaguuguauucaucUUGGCAa	3' <i>NR1H4</i> 3' prime

B

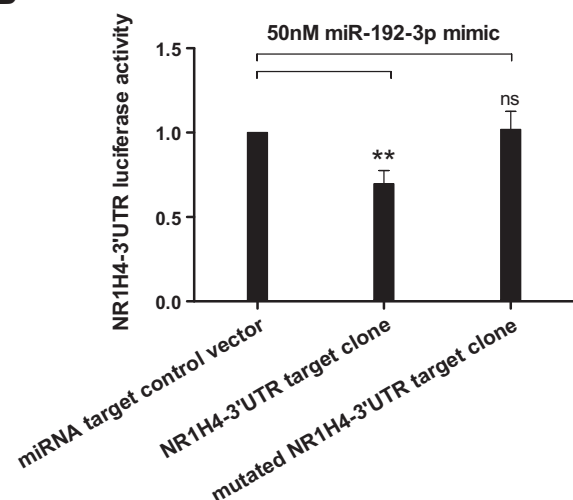


Fig. 1. A: predicted binding sites for the seed sequence of miR-192-3p in the 3'UTR of *NR1H4* mRNA using the bioinformatic tool miRANDA (www.microna.org). B: relative reporter gene activities at 24 h after cotransfection of Huh-7 cells with the wild-type *NR1H4*-3'UTR target clone or mutated *NR1H4*-3'UTR target clone. The miR target clone control vector was used for normalization. Experiments were performed as triplicates and repeated 3 times. ***P* < 0.01; ns, not significant.

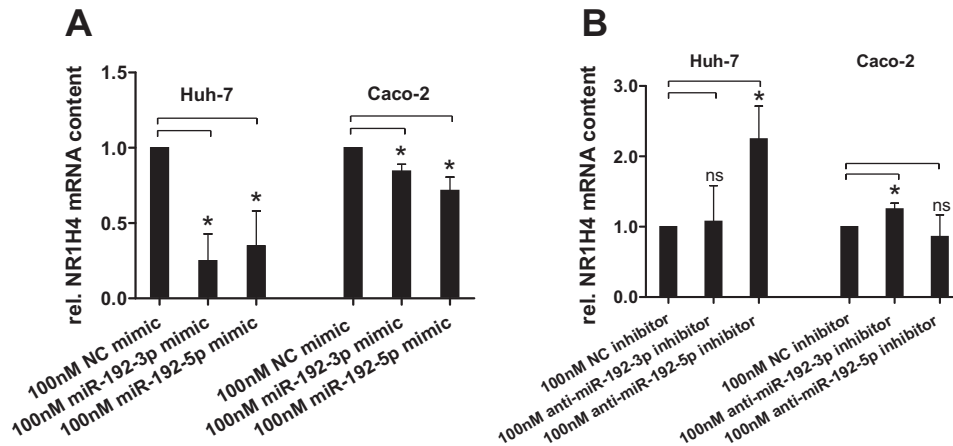


Fig. 2. Effect of miR-192-5p and -3p mimics (A) and inhibitors (B) on *NR1H4* mRNA expression in Huh-7 and Caco-2 cells at 48 h and 24 h, respectively, after transfection. *NR1H4* mRNA expression relative (rel.) to β -actin was determined by real-time PCR. The miR mimic/inhibitor negative control (NC) was used for normalization. Experiments were repeated 3 times. * $P < 0.05$; ns, not significant.

352 (position relative to translational stop codon, NG_029843.1, Fig. 1A). No binding site within the *NR1H4* 3'UTR was predicted for miR-192-5p. Cotransfection of miR-192-3p mimics and the *NR1H4* 3'UTR target sequence into Huh-7 cells resulted in a 30% decrease in luciferase activity compared with the empty vector control ($P < 0.01$). In contrast, luciferase activity remained unaffected by cotransfection of the *NR1H4* 3'UTR target sequence carrying the miR-192 binding sites in the mutated form compared with the wild-type construct (Fig. 1B), indicating a negative interaction of miR-192-3p with the predicted binding sites in the *NR1H4* 3'UTR.

MiR-192 attenuates endogenous NR1H4 mRNA levels in Huh-7 and Caco-2 cells. To investigate the effect of miR-192 on endogenous FXR expression levels, Caco-2 and Huh-7 cells were transfected with 100 nM miR-192-3p or -5p mimics for

24 or 48 h. As shown in Fig. 2A, *NR1H4* mRNA levels were repressed to 75 and 65% by miR-192-3p and -5p mimics, respectively, in Huh-7 cells ($P < 0.05$). In Caco-2 cells, a decrease by 15 and 28% in *NR1H4* mRNA expression was detected after transfection with miR-192-3p and -5p mimics for 24 h ($P < 0.05$). To examine whether transfection of an antagomir could reverse the endogenous miR-192-dependent inhibitory effect on FXR expression, Caco-2 and Huh-7 cells were transfected with 100 nM anti-miR-192 inhibitors for 24 or 48 h, respectively. As shown in Fig. 2B, *NR1H4* mRNA levels were increased significantly by anti-miR-192-3p inhibitors in Caco-2 cells as well as anti-miR-192-5p inhibitors in Huh-7 cells compared with the anti-miR miRNA inhibitor negative control ($P < 0.05$). Thus the miR-192-dependent effect on FXR regulation appeared to be stronger in Huh-7 cells compared with Caco-2 cells.

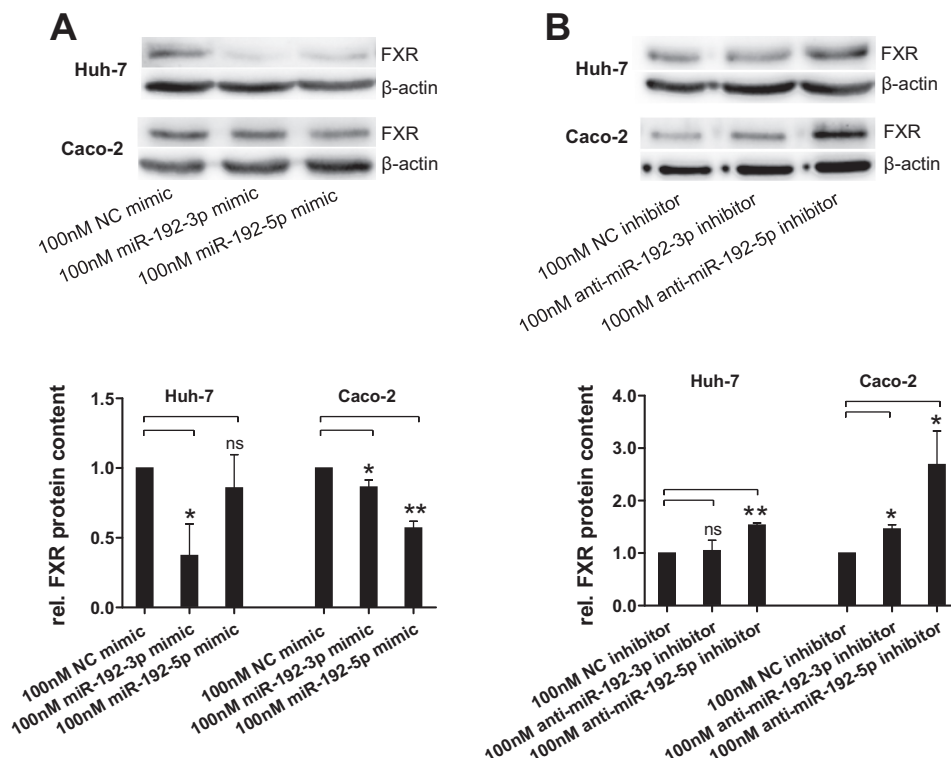


Fig. 3. Effect of miR-192-5p and -3p mimics on FXR protein expression in Huh-7 cells at 72 h after transfection and in Caco-2 cells at 48 h after transfection (A). Anti-miRNA-dependent effect on FXR protein levels (B). Caco-2 and Huh-7 cells were treated with anti-miR-192-5p and -3p inhibitors for 48 and 72 h. FXR protein expression relative to β -actin was determined by Western blotting and densitometry. Bands of 1 representative blot are shown for each condition and cell line. Experiments were repeated 3 times. ** $P < 0.01$, * $P < 0.05$; ns, not significant.

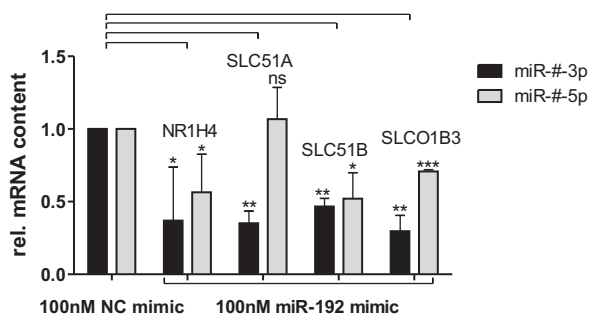


Fig. 4. Effect of miR-192-5p and -3p mimics on mRNA expression of key FXR target genes important for bile acid homeostasis in Huh-7 cells at 48 h after transfection. *SLCO1B3*, *SLC51A*, and *SLC51B* mRNA expression relative to β -actin was determined by real-time PCR. The miR mimic negative control (NC) was used for normalization. Experiments were repeated 4 times. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; ns, not significant.

MiR-192 suppresses FXR protein translation in Huh-7 and Caco-2 cells. Consistent with the miRNA-dependent effects on *NR1H4* mRNA expression, a 63% decrease in FXR protein expression was seen after transfection with miR-192-3p mimic in Huh-7 cells ($P < 0.05$). Weaker downregulation by 14% was observed after transfection of miR-192-5p. A decrease by 14 and 43% in FXR protein expression was observed in Caco-2 cells after transfection with miR-192-3p and -5p, respectively (Fig. 3A). As shown in Fig. 3B, the endogenous miR-192-dependent suppressive effect on FXR protein expression was reversed upon transfection with anti-miR-192-3p or -5p inhibitors. In Huh-7 cells, an increase in FXR protein levels was not seen following transfection of the miR-192-3p antagomir, which could be explained by the lower expression of the 3' in relation to the 5' strand in Huh-7 compared with Caco-2 cells.

MiR-192 suppresses the expression of key FXR target genes. The bile acid transporters organic-anion transporting polypeptide 1B3 (OATP1B3, *SLCO1B3*) and organic solute transporters α/β (OST alpha/beta, *SLC51A/B*) were chosen as model genes to examine the effect of miR-192 on FXR-regulated expression of transport proteins. Huh-7 cells were transfected with 100 nM miR-192-3p or -5p for 48 h. At 24 h after transfection, cells were treated with 50 μ M CDCA for 24 h to activate FXR and compared with the miRNA mimic negative control. As shown in Fig. 4, *SLCO1B3*, *SLC51A*, and *SLC51B* mRNA levels were decreased by either one or both strands of miR-192, suggesting a reduction of gene transcription secondary to reduced expression of the transcriptional activator FXR. This was confirmed by simultaneous FXR knockdown and miRNA transfection experiment (data not shown).

Linear regression analyses reveal an inverse association between the expression levels of miR-192 and FXR in colonic adenocarcinoma. To investigate whether the in vitro effects of miR-192 on FXR expression were reproducible in vivo, we analyzed 27 tissue samples from chemotherapy-untreated patients with primary colonic adenocarcinoma (Table 2). As shown in Table 3 and Fig. 5, a significant inverse association in expression was observed for the *NR1H4* mRNA transcript coding for FXR α 2(+) and hsa-miR-192-3p. FXR α 2 isomers represent the most abundantly expressed FXR protein forms in colonic tissue, whereas the isomer FXR α 1 is predominantly expressed in liver (30). No significant associations were found for *NR1H4* mRNA and hsa-miR-192-5p expression, showing

Table 2. Clinical characteristics of 27 patients with primary colonic adenocarcinoma

Parameters	Category	N (%)
Gender	male	15 (55.6)
	female	12 (44.4)
AJCC stage	pT1	6 (22.2)
	pT2	13 (48.2)
	pT3	4 (14.8)
	pT4	4 (14.8)
Tumor grade	1	3 (11.1)
	2	20 (74.1)
	3	4 (14.8)
Treatments	chemotherapy-naive	27 (100)

Clinical characteristics of the 27 patients showing primary colonic adenocarcinoma who were included into linear regression analysis. AJCC, American Joint Committee on Cancer.

that the 5' strand does not confer pronounced *NR1H4* mRNA degradation in vivo. These findings support the regulatory effects of miR-192 on FXR expression in hepatoma and colon cancer-derived cell lines observed in vitro.

MiR-192 exhibits suppressive effects on proliferation of Huh-7 and Caco-2 cells. To investigate the functional significance of the observed miR-192/FXR interaction, we performed cell proliferation assays using alamarBlue. As shown in Fig. 6, transfection with miR-192-3p mimic significantly reduces proliferation of Huh-7 and Caco-2 cells, whereby a stronger effect could be observed in the hepatoma cell line. These findings confirm the previously described suppressive effects of miR-192 on proliferation of the colon cancer cell lines HT-29, RKO, and HCT116 (27). Knockdown of FXR expression causes similar antiproliferative effects in Huh-7 cells. In contrast, reduction in FXR expression does not seem to have any influence on the proliferative potential of Caco-2 cells. By performing cell invasion assays, we saw a trend toward a suppressive effect for both strands of the miR-192 precursor molecule on cell invasion of Huh-7 cells; however, the results were not significant (data not shown). In a previous work, Lian et al. (19) showed that miR-192 could significantly downregulate cell invasion of Huh-7 cells.

DISCUSSION

The aim of this study was to investigate whether FXR is a target of miR-dependent, posttranscriptional gene regulation. Regulation of FXR expression by miR-421 as an oncogenic miR in biliary tract cancer has already been postulated (36). Our results show an additional, as yet uncharacterized role of miR-192 in regulating FXR expression.

Table 3. Linear regression analysis of miR-192-3p expression with FXR α 2(+), FXR α 1(−), and miR-192-5p expression in primary colonic adenocarcinoma

Transcript	Coefficient	Standard Error	P Value
FXR α 2(+)	−1.57	0.66	2.59E-02
FXR α 1(−)	1.87	0.70	1.39E-02
hsa-miR-192-5p	0.31	0.14	4.13E-02

Linear regression analysis of hsa-miR-192-3p with the *NR1H4* mRNA transcripts coding for FXR α 2(+) and FXR α 1(−), and miR-192-5p expression in primary colonic adenocarcinoma tissue obtained from 27 patients. miR-192-3p inversely correlates with FXR α 2(+) expression. Adjusted R^2 : 0.348.

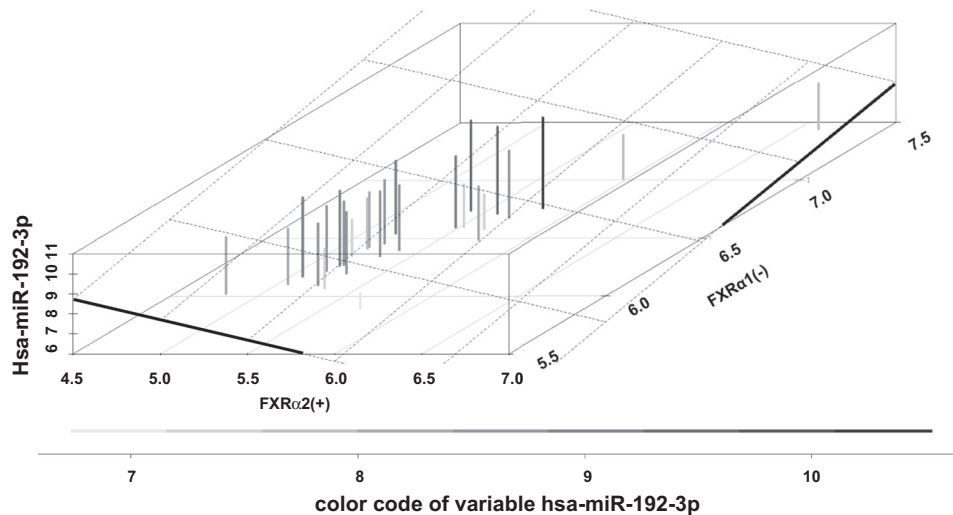


Fig. 5. Correlation between FXR and miR-192 expression in colonic adenocarcinoma-derived tissue samples from 27 patients by linear regression analysis. A significant inverse association was detected for miR-192-3p and the *NR1H4* mRNA transcript coding for FXR α 2(+) (coefficient = $-1.57/P = 0.0259$).

Our in vitro experiments elucidated the inhibitory effect of miR-192 on FXR expression by transfecting colon and liver cell lines with miR-192 mimics and inhibitors. In the case of the 3' strand, we found a significant miR-192-dependent inhibitory effect on *NR1H4*/FXR mRNA and protein expression in Huh-7 cells, whereas in Caco-2 cells only transfection with the anti-miR-192-3p inhibitor showed a relevant effect. A possible explanation for the observed weaker inhibitory effect of the miR-192-3p mimic in Caco-2 cells, compared with that in Huh-7 cells, may be the more abundant expression of miR-192 in the colon cell line. The observed miR-192*-dependent effects on *NR1H4* mRNA levels in both cell lines and our in vivo findings that showed a strong inverse miR-192* and FXR α 2(+) correlation in colonic adenocarcinoma support our hypothesis of a strong endogenous effect of miR-192 on FXR expression. Degradation of *NR1H4* mRNA transcripts can be explained by perfect complementary interference of the miR-192-3p seed sequence at the two in silico-predicted bind-

ing positions of the *NR1H4*-3'UTR. The 5' strand of miR-192 also appeared to repress FXR gene expression in vitro, albeit through a different mechanism. Transfections with the corresponding antagomirs support our findings with both mimics in our model cell lines.

According to the miRBase database, about 80 different human miRNA precursors can yield two abundant mature miRNAs, i.e., the 5' strand (miR-#-5p) and 3' strand (miR-#-3p) with different seed sequences and mRNAs as binding targets. There is increasing evidence for interplay between the 5' and 3' strands of the same precursor molecule targeting the same group of genes, thereby reinforcing a certain phenotype (10), which can be supported by our data.

Hsa-miR-192 has been shown to play a crucial role in the pathogenesis of colon carcinoma, the third most common cancer in Western countries (6). Because of its cancer stage-dependent decline in expression, miR-192 has been suggested to be a potential biomarker to predict metastasis in colon

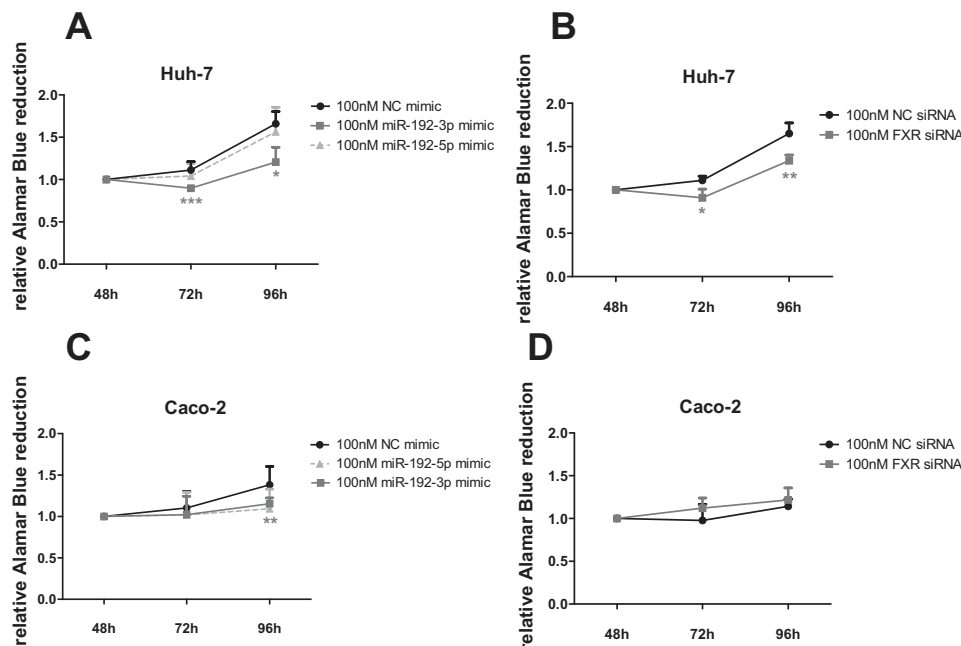


Fig. 6. Impact of miR-192-5p and -3p (A and C) or FXR siRNA (B and D) on proliferation of Huh-7 and Caco-2 cells by AlamarBlue assay. The 48-h values of each condition were used for normalization. Experiments were repeated 4 times. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

carcinoma patients (9). Our findings regarding the inverse association of FXR and miR-192* expression in colonic cancer patients suggest that this miRNA-dependent mechanism of FXR regulation could play an important role in carcinogenesis. We additionally showed that miR-192 can suppress expression of the bile acid and anticancer drug transporter OATP1B3 and the bile acid transporter OST α / β in a FXR-dependent manner.

It remains unclear whether restoration of miR-192 expression and the consequently diminished expression of FXR target genes would be beneficial for cancer patients, especially considering that miR-192 has been shown to possibly act as an oncogenic miRNA by downregulation of Smad interacting protein 1 in other inflammation-related cancers (21, 26, 35). Therefore, it can be speculated that diminished expression of the transport protein OST α /OST β , a heterodimer-forming bile acid transporter important for the excretion of bile acids from hepatocytes and enterocytes, may be a mechanism of intracellular bile acid accumulation promoting inflammation and/or cancer development in certain cases.

By performing cell proliferation and invasion assays, we were able to confirm the previously described suppressive role of miR-192 in liver and colon cancer progression. We observed a miR-192-associated antiproliferative effect in Huh-7 cells that is stronger than in Caco-2 cells, where no noteworthy effects of the 3' strand arm could be seen on FXR protein levels. Furthermore, knockdown of FXR did not show any influence on Caco-2 cell proliferation. These observations support the hypothesis that the antiproliferative effect of miR-192-3p may be to some extent FXR-dependent. Future studies have to elucidate to what extent the miR-192/FXR interplay supports or inhibits tumor pathogenesis. Our results are in line with reports showing that enhanced FXR expression and the associated altered expression of FXR-regulated drug-uptake transporters is related to chemoresistance in cancer patients. Treatment of the colorectal adenocarcinoma cell line LS174T with cisplatin leads to a cisplatin-resistant phenotype that is accompanied by a 350-fold increase in FXR expression (22). Furthermore, increased expression of OATP1B3 has been suggested to confer antiapoptotic resistance to paclitaxel by altering p53-dependent pathways (18).

It may be of value to confirm the detected association between miR-192 and FXR expression in larger cohorts. We cannot exclude the contribution of other miRs or epigenetic factors to FXR regulation. However, a further step would be to systematically test all in silico-predicted miRs for effects on the *NR1H4*-3'UTR.

In conclusion, miR-192-5p and -3p negatively regulate the expression of FXR in a synergistic manner, thereby significantly decreasing the expression of FXR target genes OST α / β and OATP1B3. We show a new miR-dependent mechanism of FXR regulation, which could affect the expression of FXR target genes and plays a role in the pathogenesis of liver and colon cancers and their response to anticancer therapies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

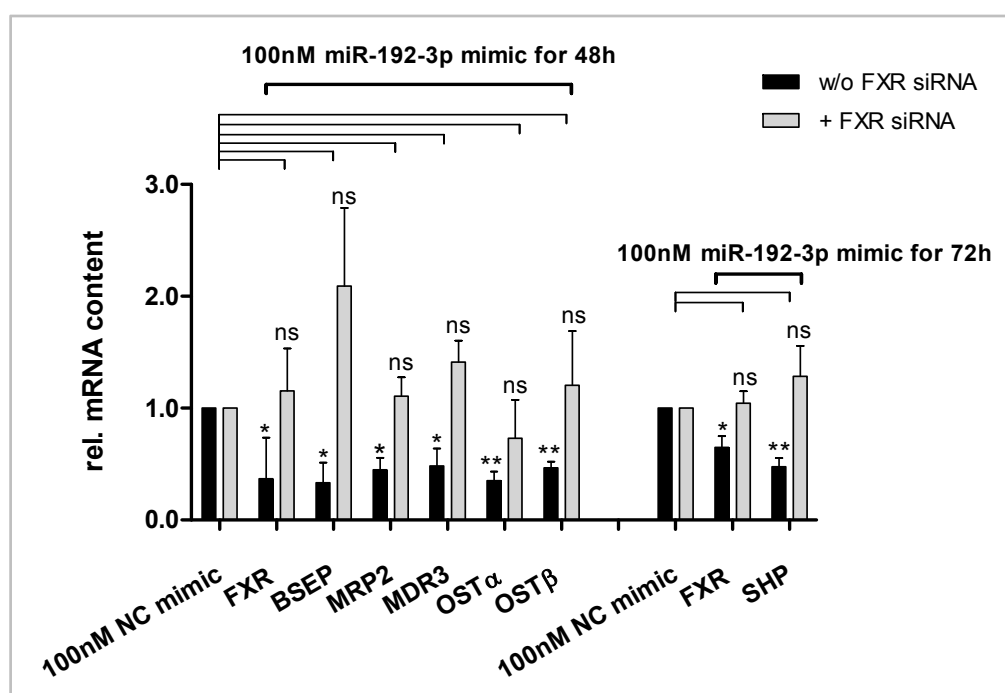
R.K., J.M., and G.A.K.-U. conception and design of research; R.K. performed experiments; R.K., A.B., and W.E.T. analyzed data; R.K. interpreted results of experiments; R.K. prepared figures; R.K. drafted manuscript; H.B.S., J.M., and G.A.K.-U. edited and revised manuscript; G.A.K.-U. approved final version of manuscript.

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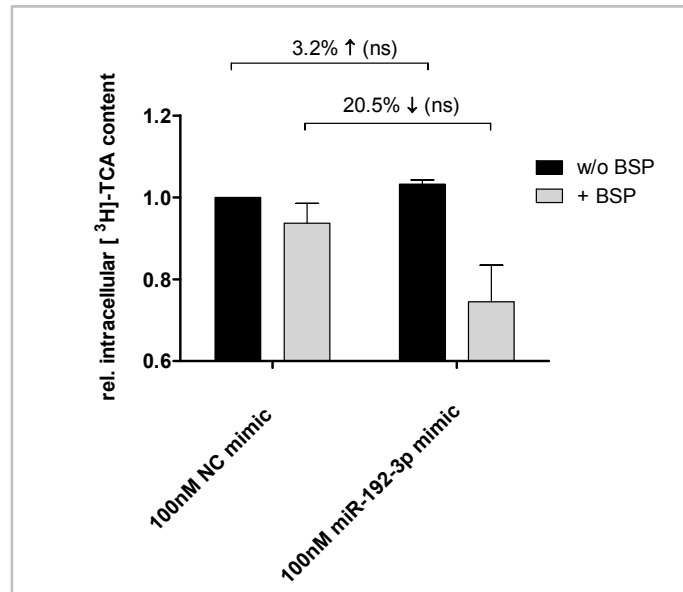
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SUPPLEMENTARY FIGURES



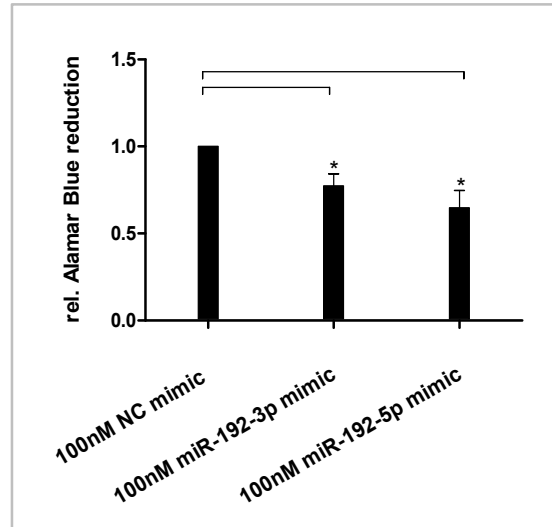
Suppl. Figure 1: MicroRNA-192-3p potentially impacts intracellular bile acid homeostasis in Huh-7 cells. MicroRNA-192-3p mimic significantly suppressed the mRNA expression of FXR key target genes, which are important in the hepatic, anticholestatic compensatory response. The observed miR-dependent suppressive effect on the expression of the bile acid transporters BSEP, MRP2, MDR3, OST α/β and on the expression of the nuclear receptor SHP occurred secondary to the reduced expression of the transcriptional activator FXR (confirmed by simultaneous FXR knockdown and miRNA transfection: 100 nM siRNA, miRNA or negative control for 48 or 72 h). mRNA levels relative to β -actin were determined by real-time PCR. The miR mimic negative control (NC) was used for normalization. 24 h after transfection, cells were treated with 50 μ M CDCA for 24 h and 48 h, respectively, to activate FXR. Experiments were repeated three times and one sample t-test was used for statistical analysis. ** $p < 0.01$; * $p < 0.05$; ns, not significant.



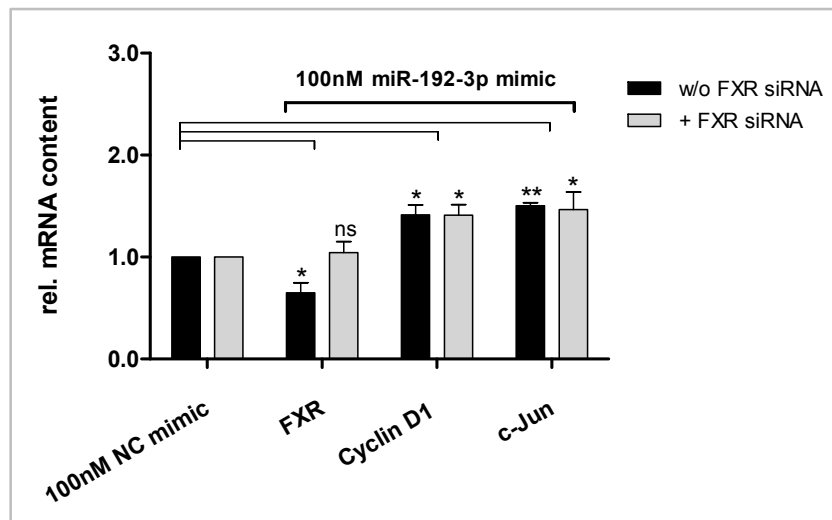
Suppl. Figure 2: Taurocholate (TCA) transport is influenced by miR-192-3p transfection in Huh-7 cells.

To study the functional relevance of the observed miR-192-dependent suppressive effect on BSEP expression (Suppl. Fig. 1), intracellular TCA concentrations were measured 72 h after miRNA transfection, 2 h after TCA incubation and 30 min after bromsulphthalein (BSP) treatment. BSP is a potent inhibitor of the hepatic basolateral bile acid transport by repressing OATP and NTCP activities. As NTCP is not expressed in Huh-7 cells (data not shown), the BSP-dependent effect observed in this experiment can be attributed to OATP inhibition. Due to the moderate miR-192-dependent decrease of intracellular TCA levels (20.5%) in BSP-treated cells, we conclude that other BSEP-independent, miR-192-3p-suppressed bile acid transporters are involved in TCA transport. As miR-192 did not influence the expression of OATP1B1 and as BSEP expression is overall low in Huh-7 cells (data not shown), OATP1B3 is probably the predominant TCA transporter in this cell line. To activate FXR, 24 h after transfection, cells were additionally treated with 50 μ M CDCA for 48 h. The experiment was run in duplicates and the miR mimic negative control (NC) w/o BSP was used for normalization. ns, not significant.

Suppl. Method to Figure 2: 72 h after miRNA transfection, cells were incubated for 2 h with 10 μ M TCA (cold TCA mixed 3:1 with [3 H]-TCA (15.4 Ci per mmol, Perkin-Elmer)) and half of the conditions additionally with 10 μ M BSP for 30 min. After four washing steps, cells were lysed with 1% Triton and 150 μ l of the lysate were measured together with 4 ml Ultima-GoldTM scintillation reagent (Perkin-Elmer) using the TriCarb Liquid Scintillation Analyzer (Packard). Count per minutes (cpm)-values were adjusted to the relative protein concentrations.



Suppl. Figure 3: miR-192-3p and -5p significantly decrease the viability of Huh-7 cells in a FXR-independent manner. Cell viability was measured 48 h after transfection using Alamar Blue assay. No alterations in cell viability were observed after cell treatment with 100 nM FXR siRNA for 48 h (data not shown). The miR mimic negative control (NC) was used for normalization. Experiments were repeated three times and one sample t-test was used for statistical analysis. * $p < 0.05$.



Suppl. Figure 4: FXR-independent effects of miR-192-3p on cyclin D1 and c-jun expressions in Huh-7 cells. This experiment was performed to study the effect of the miR-192/FXR interplay on target genes known to play a crucial role in cell cycle regulation. 72 h after transfection, miR-192-3p significantly induced mRNA expression of cyclin D1 and c-jun, but, in a FXR-independent manner. A significant increased expression of cyclin D1 and c-jun was also observed after cell transfection of miR-192-5p for 48 h (data not shown). Enhanced cyclin D1 and c-jun expressions are both important signs for endogenous cell cycle activation and subsequent apoptosis or cell proliferation, dependent on concomitant stimuli. 24 h after transfection, cells were treated with 50 μ M CDCA for 48 h to activate FXR. mRNA levels relative to β -actin were determined by real-time PCR. The miR mimic negative control (NC) was used for normalization. Experiments were repeated three times and one sample t-test was used for statistical analysis. ** $p < 0.01$; * $p < 0.05$; ns, not significant.

IV. CHAPTER 3

The organic anion transporting polypeptide OATP1B3 is regulated by microRNA-509 and microRNA-656

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Draft manuscript

Abstract

Aims. OATP1B3 mediates the uptake of endogenous and xenobiotic compounds from portal venous blood into the liver. Substrates of OATP1B3 include anti-cancer agents and other drugs. The high inter-individual variability in OATP1B3 expression, which is thought to impact on drug safety and efficacy, cannot be explained by genetic polymorphisms alone. In the current study, we investigated the extent to which microRNAs regulate the expression of OATP1B3 (*SLCO1B3* gene).

Main Methods. 21 miRNAs, predicted *in silico* to putatively bind to *SLCO1B3*, were tested for their regulatory effect on gene expression in luciferase gene reporter assays co-transfecting them with *SLCO1B3* 3'-UTR subcloned into pmirGLO luciferase vector. Mutations were inserted into the consensus sequences for miR-509-3p or miR-656-3p that showed the strongest effect on luciferase activity. *SLCO1B3* mRNA and protein were measured by real-time PCR and western blot analysis in Huh-7 cell extracts treated with miR-509-3p, miR-509-5p and miR-656 mimics. Correlation between *SLCO1B3* mRNA and miR-509-3p, miR-509-5p and miR-656 expression was analyzed in human intrahepatic cholangiocarcinoma samples using Pearson's and Spearman's correlation analyses.

Key findings. Luciferase activity was site-specifically and significantly decreased in co-transfection experiments with miR-509-3p or miR-656 mimics. MiR-509 and miR-656-3p mimics caused decreased levels of *SLCO1B3* mRNA in Huh-7 cells, leading to significantly suppressed levels of OATP1B3 protein compared with mock transfected cells. In human cholangiocarcinoma, expression of *SLCO1B3* mRNA was inversely correlated with miR-509-5p ($p < 0.05$).

Conclusions. In summary, we show that the microRNAs miR-509 and miR-656 regulate OATP1B3 expression *in vitro* and *in vivo*.

Introduction

The hepatic transporter organic anion-transporting polypeptide 1B3 (OATP1B3) mediates the uptake of many endogenous and exogenous compounds from portal venous blood into the liver. OATP1B3 and its close family member OATP1B1 share 80% sequence homology and show a partly overlapping substrate spectrum. Important substrates of the OATP family comprise bile salts and hormones as well as drugs such as statins, antidiabetics, and several anti-cancer drugs including tyrosine kinase inhibitors (i.a. sorafenib), platinum drugs,

paclitaxel or SN-38, the active metabolite of irinotecan [1-5]. Several genetic polymorphisms in *SLCO1B1* have been described, which appear to predispose to altered pharmacokinetics and an elevated risk for adverse drug reactions [6-8].

Genetic variants of *SLCO1B3* (the gene encoding OATP1B3) have also been detected and studied, but the relevance of these polymorphisms for drug safety and efficacy has not yet been clearly established [9]. A total deficiency of OATP1B1 and OATP1B3, induced by loss of function mutations, leads to Rotor Syndrome, a benign accumulation of bilirubin glucuronide in the blood associated with jaundice [10]. OATP1B3 displays a high variance in hepatic protein expression that varies between 4.9 and 30 fmol/μg protein (range of mean expression 1.1 to 6.5 fmol/μg protein) [11]. Several studies have shown that high variance of OATP1B3 expression in healthy liver tissue and in hepatocellular carcinoma contributes to the variability in measured plasma levels of drugs such as atorvastatin or the liver-specific contrast agent gadoxetic acid [12,13]. Teft and colleagues reported that the progression-free survival of colorectal carcinoma under irinotecan therapy correlated significantly with the expression of OATP1B3 in tumor tissue, underlining the importance of OATP1B3 expression for the response to cancer therapy [14].

The exact molecular mechanisms regulating OATP1B3 expression have not been well studied. Imai et al. demonstrated in different cancer cell lines that OATP1B3 expression is partly driven by methylation patterns in near proximity to the transcription start site [15]. MicroRNAs (miRNAs, miRs) are small molecules of approximately 20 bp that bind to the 3' untranslated regions (UTRs) of target genes and thus inhibit mRNA translation or even lead to mRNA degradation. Several drug transporters and drug metabolizing enzymes are regulated by miRNAs. CYP3A4, the activity of which shows little modulation due to genetic polymorphisms, is regulated by miR-27b and miR-298 [16]. The hepatic export pump MRP2 appears to be regulated by miR-379, which modulates the expression of this transport protein in a rifampicin-dependent manner [17]. No study has yet addressed the extent to which OATP1B3 expression is directly regulated by miRNAs. Investigating this regulatory mechanism may help explain the observed inter-individual and tissue-dependent variability of OATP1B3 expression, and may widen our current understanding of the safety and efficacy of therapy with OATP1B3 substrates.

In this study, we investigated to what extent miRNAs can modulate the hepatic expression of OATP1B3. We focused on miRNAs miR-509 and miR-656 (which we predicted *in silico* as

binding to OATP1B3) and studied their impact on OATP1B3 expression in Huh-7 cells and in array data of human cholangiocarcinoma samples.

Materials and Methods

Cell culture. Human hepatoma derived Huh-7 cells (American Type Culture Collection, Molsheim, France) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were kept at 37°C in an atmosphere containing 5% CO₂.

***In silico* prediction of putative miRNA binding sites in *SLCO1B3*.** Putative binding sites for miRNAs within the 3' UTR of *SLCO1B3* mRNA were predicted using the bioinformatics tools TargetScan (<http://www.targetscan.org/>), miRANDA (<http://www.microrna.org>) and MicroCosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>). Based on the prediction of putative binding to *SLCO1B3*, initially, 21 miRNAs were selected for a further analysis in luciferase gene reporter assays. MiRNAs were tested in gene reporter assays, if a binding was predicted with at least two different bioinformatics tools. Additionally, miRNA-509 (predicted with MicroCosm) was included in subsequent investigations as well due to its putative role in liver associated malignant diseases.

Cloning of reporter gene constructs and mutagenesis reactions. To confirm the impact of miRNAs on gene expression, luciferase gene reporter assays were performed. The 3' UTR of *SLCO1B3* was subcloned into pmirGLO vector (Promega, Fitchburg, WI, USA) using primers listed in Table 1 and the restriction enzymes NheI and XbaI (Thermo Scientific, Waltham, MA, USA). Recognition sites for the restriction enzymes are underlined in Table 1. The pmirGLO reporter vector carries both the firefly and *Renilla* luciferase reporter genes, which allows a direct normalization of the obtained luciferase results without any additional transfection of a second reporter vector.

MiRNAs miR-509-3p and miR-656-3p were detected to significantly downregulate luciferase gene reporter activity, To further test the functional relevance of the predicted binding sites for these two miRNAs (Fig. 1), the consensus sequences detected *in silico* were artificially mutated using mutagenesis primers (Table 1) and the QuikChange II XL Site-Directed

Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The mutations were confirmed by DNA sequencing (Microsynth, Balgach, Switzerland). Inserted mutations are marked in bold and are underlined in Table 1.

Table 1: Primers used for subcloning and mutagenesis reactions.

Primer name	Direction	Sequence	Enzyme/miRNA	Application
1B3_3UTR_FW2	Forward	5'-aggctagccattgcattgattcattaagatg	NheI	Subcloning of <i>SLCO1B3</i> 3'-end into pmirGlo
1B3_3UTR_RV2	Reverse	5'-agtctagaatttctgaaaaatacaacttaac	XbaI	Subcloning of <i>SLCO1B3</i> 3'-end into pmirGlo
hsa-miR-509-3p_F	Forward	5'-attccaacattcttccgttctgcccagcgaaggataagctatg	miR-509-3p	Mutagenesis
hsa-miR-509-3p_R	Reverse	5'-catagacttatccatcgctgcgcagaaacggaagaatgttggaat	miR-509-3p	Mutagenesis
hsa-miR-656_F	Forward	5'-ccaaaatctggcctgggtgttccgacaatatatatttcatgtt	miR-656-3p	Mutagenesis
hsa-miR-656_R	Reverse	5'-aacatgaaaatatattgtcggaaacacccaggccagattttgg	miR-656-3p	Mutagenesis

Luciferase gene reporter assays. Luciferase gene reporter assays were performed by cotransfecting wild type and artificially mutated constructs into Huh-7 cells. Cells were seeded into 48-well plates at a density of 1.6×10^5 cells/ml per well. 24 h later, cells were transfected with 25 nM hsa-mirVANA[®] mimics to miRNAs or Negative Control #1 miRNA mimic (Life Technologies, Carlsbad, CA, USA) together with 100 ng of wild type, mutated or empty pmirGLO reporter construct using Lipofectamine[®] 2000 (Invitrogen, Carlsbad, CA, USA) and Opti-MEM I medium (Invitrogen). The activities of firefly and *Renilla* luciferase were measured 48 h after cotransfection using the Dual-Luciferase[®] Reporter 1000 Assay System (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol. Analysis was performed using the GloMax[®] Multi Detection System (Promega). Each luciferase gene reporter assay using wild type and mutated target clones was performed at least in triplicate.

Transient transfections of miR-509-3p and miR-656-3p and real-time PCR. Based on the results obtained in luciferase gene reporter assays and in mRNA/miRNA correlation analyses of intrahepatic cholangiocarcinoma (ICC) tissue samples (described below), we further studied, to what extent miR-509-3p, miR-509-5p and miR-656-3p influence the endogenous expression of OATP1B3. Huh-7 cells were transiently transfected with the respective miRNA

mimics and *SLCO1B3* mRNA levels were determined by real-time reverse transcription polymerase chain reaction (RT-PCR). Cells were seeded into 12-well plates at a density of 8×10^4 cells/ml and 24 h later transfected with 100 nM hsa-mirVANA[®] miR-509-3p, miR-509-5p, miR-656-3p or Negative Control #1 using Lipofectamine[®] RNAiMax (Invitrogen). Cells were harvested after 48 h and 72 h for the extraction of total mRNA using Trizol[®] reagent (Life Technologies) and after 72 h for the extraction of protein using RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate (all from Sigma-Aldrich), 1 mM EDTA (Pharmacia Biotech, Uppsala, Sweden), 0.1% SDS (Sigma-Aldrich), 10% glycerol (Sigma-Aldrich), made up to 1000 ml with ddH₂O)). Three independent experiments were performed.

For the performance of RT-PCR, 1.5 µg RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Waltham, MA, USA). For *SLCO1B3* mRNA expression analysis, 2 µl of 1:5 diluted cDNA samples and 8 µl of *SLCO1B3*-specific RT-PCR Universal Fast Master Mix were mixed together (TaqMan[®] Gene Expression Assays, Life-Technologies). β -actin (*ACTB*) mRNA was measured as an internal control and used to normalize the RT-PCR results. All measurements were performed in triplicate.

Western blot analysis. Huh-7 cell-derived protein extract (5 and 8 µg) was diluted 1:5 in loading buffer, denatured and loaded on an 8% SDS-polyacrylamide gel. The separated samples were subsequently electroblotted on polyvinylidene fluoride membranes, which were blocked for 1 h in 5% (wt/vol) non-fat milk/PBS-Tween 20 (0.1% (vol/vol) Tween 20 in PBS; PBS-T) blocking solution. Anti-OATP1B3 serum or anti- β -actin antibody (Abcam, Cambridge, UK) was then added and membranes were incubated for 16 h or 1 h, respectively, at 4°C. Membranes were washed three times with PBS-T and once with blocking solution, and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit, Thermo Scientific) for 1 h at room temperature. After a second round of four washing steps, the bands were visualized and quantified using SuperSignal[®] West Femto Maximum Sensitivity Substrate (Thermo Scientific) and the Fusion FX luminescence detector system (Vilber Lourmat, Marne-La-Valle, France). The measured intensities of β -actin bands were used to normalize the results for OATP1B3 expression. The experiments were repeated three times using three different batches of Huh-7 cells.

MessengerRNA/miRNA correlation analyses in cholangiocarcinoma probes. To investigate the impact of miR-509 and miR-656 on the expression of *SLCO1B3* *in vivo*, Pearson's and Spearman's correlation analyses were performed between the expression of these miRNAs and *SLCO1B3* in human intrahepatic cholangiocarcinoma samples (E-GEOD-32958, ArrayExpress database (<https://www.ebi.ac.uk/arrayexpress/>)). A panel comprising 20 cholangiocarcinoma (CCA) samples, i.e. 14 intrahepatic cholangiocarcinoma (ICC) and 6 combined hepatocellular and cholangiocarcinoma (CHC) samples was investigated with regard to these correlations. Expression data were obtained using the Affymetrix Human Exon 1.0 ST Array (Affymetrix, mRNA determination) and the Nanostring nCounter Human microRNA Expression platform (miRNA determination) [18].

Statistical analyses. One sample t-tests were performed to compare the effects of miRNA mimics on luciferase gene reporter activity and on endogenous mRNA and protein expression of OATP1B3. Results are shown as average values (+/-) standard deviation (SD). In these analyses, as well as in the Pearson's correlation and Spearman's Rho analyses, p-values ≤ 0.05 were considered to be statistically significant. Statistical analyses were performed using R (<http://www.r-project.org/>, version 2.15.2) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA; www.graphpad.com, version 5.04).

Results

MiR-509-3p and miR-656-3p decrease OATP1B3 mRNA and protein by binding to consensus sequences within the 3' UTR of *SLCO1B3*. A bioinformatic investigation of the 3' UTR of *SLCO1B3* with three different bioinformatic tools resulted in the prediction of putative binding sites for 21 different miRNAs including miR-509-3p and miR-656-3p (Fig. 1). Cotransfection experiments in Huh-7 cells revealed strongest downregulations of luciferase activity with miR-509-3p mimic and miR-656-3p mimic, leading to a decrease in luciferase activity of 85% and 49%, respectively. Subsequent luciferase gene reporter experiments focusing on these two miRNAs showed a reconstitution of luciferase activity when using pmirGLO-constructs carrying artificially mutated binding sites for miR-509-3p and miR-656-3p mimics in cotransfection experiments (Fig. 2).

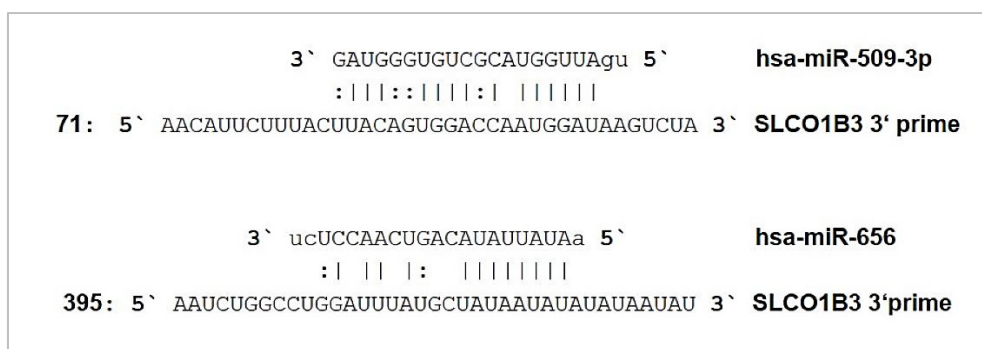


Figure 1: *In silico* predicted binding sites for miRNA-509-3p and miRNA-656-3p within the 3' UTR of *SLCO1B3* mRNA. Predictions were performed using the bioinformatics tools MicroCosm (miR-509-3p) and miRANDA (miR-656-3p, microRNA.org).

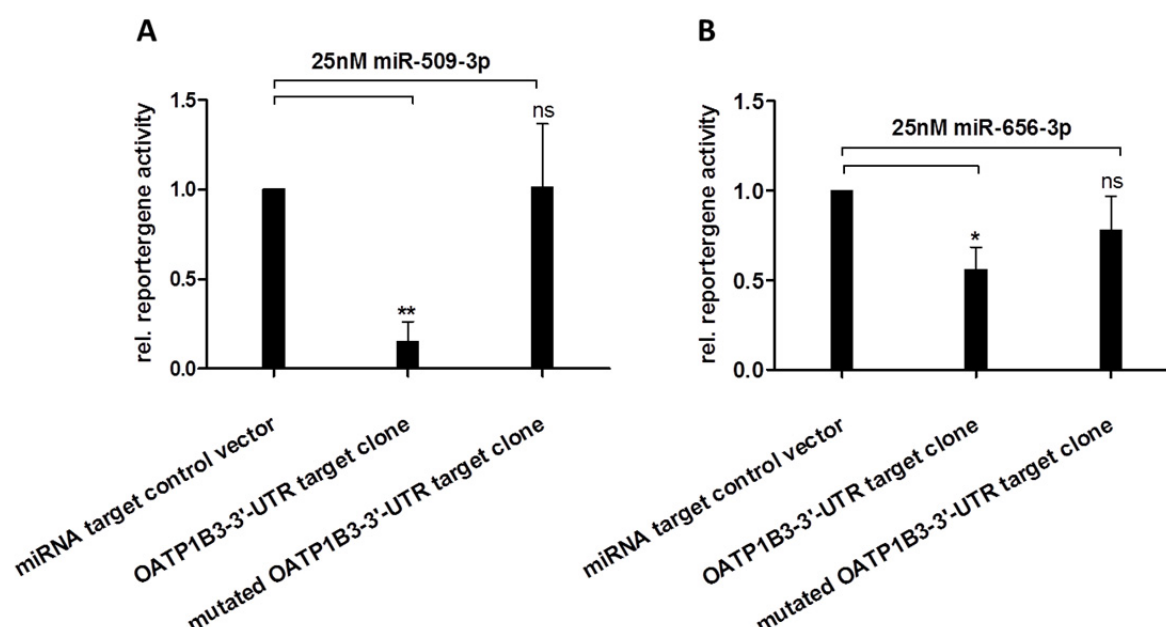


Figure 2: Relative reporter gene activities measured in Huh-7 cells 48 h after cotransfection of a *SLCO1B3*-3' UTR carrying pmirGLO vector and miRNA-509-3p (A) or miRNA-656-3p (B). Cotransfections were performed with pmirGLO-wild type clones or pmirGLO-constructs carrying the putative binding sites for the respective miRNAs in mutated form. Reporter gene activities measured in mock transfected cells were defined as 1. Reporter gene activities were compared with miRNA-mock-transfected cells. Measurements were repeated in triplicate. The figure shows the average result of three independent experiments. *p < 0.05; **p < 0.01; ns, not significant.

MiR-509-3p, miR-509-5p and miR-656-3p significantly downregulate *SLCO1B3* mRNA in Huh-7 cells. To investigate to what extent miR-509 and miR-656 inhibit OATP1B3 expression at the mRNA level, i.e., by degrading the *SLCO1B3* mRNA, Huh-7 cells were transfected with mimics to the miRNA molecules miR-509-5p, miR-509-3p, miR-656-3p or negative control. The impact of miR-509-5p on OATP1B3 mRNA and protein expression was

investigated here as well, based on significant results in correlation analyses obtained in ICC as described below. MessengerRNA amounts of OATP1B3 were determined 48 and 72 hours later. As shown in Figure 3A to C, all three miRNA mimics (i.e., miR-509-3p, miR-509-5p and miR-656-3p) decreased *SLCO1B3* mRNA levels. *SLCO1B3* mRNA was significantly decreased after 48 h by 41% (miR-656-3p), 54% (miR-509-3p) and 61% (miR-509-5p). Compared to miR-656-3p the effect of miRNA-509 on *SLCO1B3* mRNA expression was even more pronounced as reflected by a strong and significant downregulatory effect of 74% (miR-509-3p) and 61% (miR-509-5p), respectively, observed after 72h.

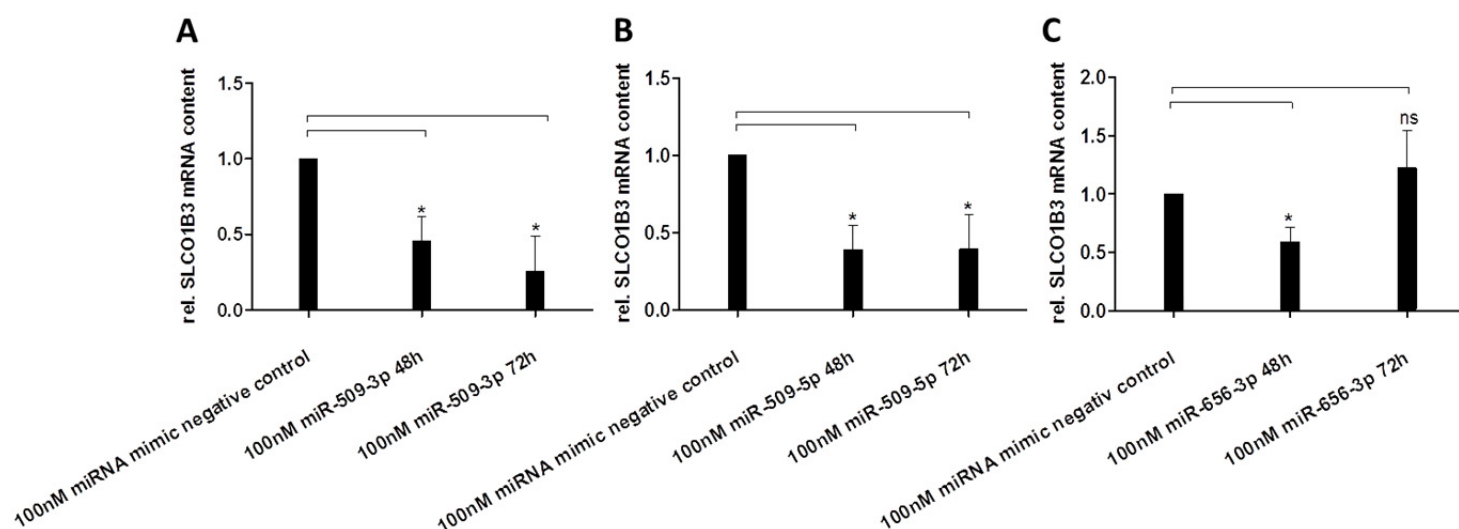


Figure 3: Effect of miR-509-3p (A), miR-509-5p (B) and miR-656-3p (C) on the endogenous expression of *SLCO1B3* mRNA in Huh-7 cells. Huh-7 cells were transfected with the respective miRNA mimics or negative control. Cells were harvested after 48 and 72 hours. *SLCO1B3* mRNA expression levels were determined by RT-PCR and normalized to β -actin (*ACTB*). A significant downregulation of *SLCO1B3* mRNA was seen for all three miRNAs after 48 h and for the miR-509 molecules after 72 h. Expression levels measured in the mock-transfected cells were defined as 1. Significance outcomes in the figure describe the pairwise comparison to mock transfected cells. The figure shows the average result of three independent experiments. * $p < 0.05$; ns, not significant.

OATP1B3 protein expression is significantly reduced in Huh-7 cells upon treatment with miR-509 and miR-656-3p. To investigate to what extent miR-509 and miR-656 influence OATP1B3 protein expression, Huh-7 cells were transfected with the respective mimics to miR-509-5p, miR-509-3p, and miR-656-3p and protein levels of OATP1B3 were determined by western blot analysis. As shown in Figure 4A to C, miR-509-3p, miR-509-5p and miR-656-3p also decreased OATP1B3 protein expression by 49%, 23%, and 50%, respectively, in Huh-7 cells (as shown by the quantification data at the bottom of the figure).

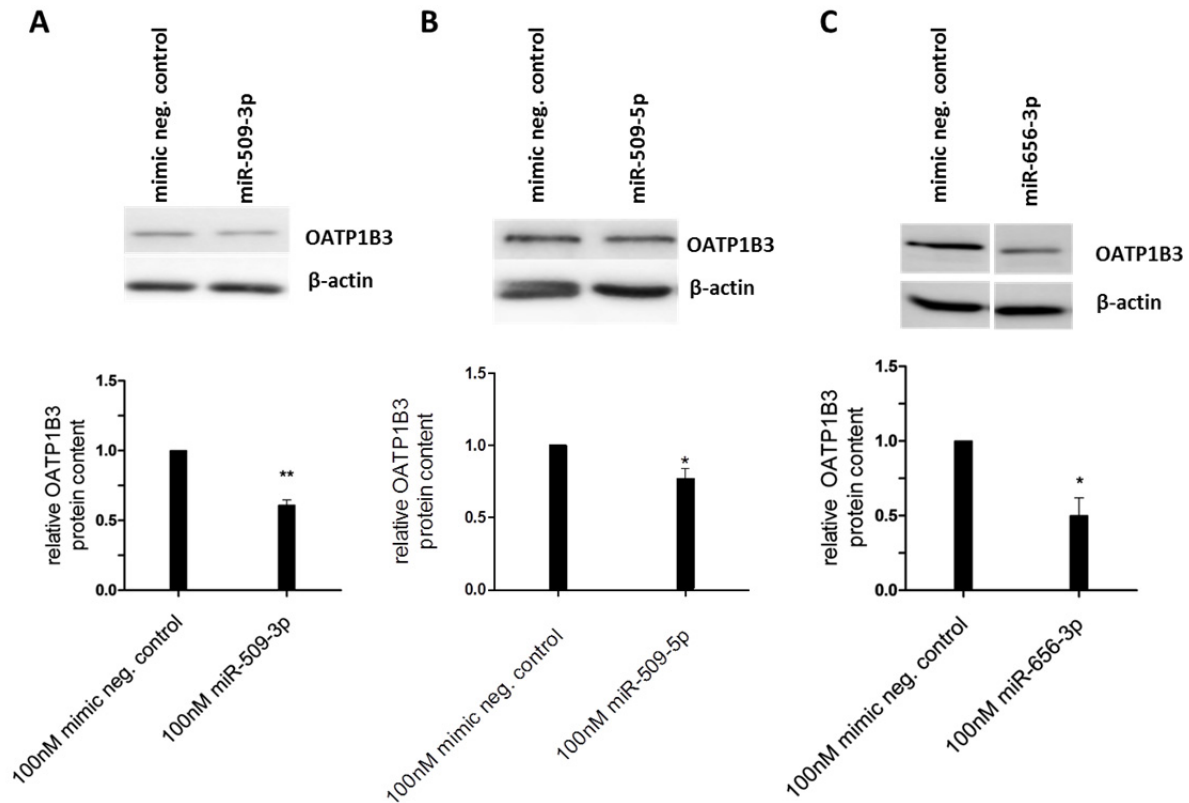


Figure 4: MiRNAs 509-3p (A), 509-5p (B) and 656-3p (C) suppress OATP1B3 protein expression levels in Huh-7 cells. Cells were transfected with mimics corresponding to the three miRNA molecules or miRNA negative control. Cells were harvested 72 h later and protein was extracted. Western blot analyses show the expression level of OATP1B3 in comparison to β -actin and in response to the miRNA transfections performed. Band intensities were quantified and are shown at the bottom of the figure. Blots show a representative result of three independent experiments performed, and the quantification is an average of three independent blots. ** $p < 0.01$; * $p < 0.05$.

Expression of miRNA-509-5p inversely correlates with the expression of OATP1B3 in intrahepatic cholangiocarcinoma. To investigate to which extent the effects of miR-509 and miR-656 on OATP1B3 expression observed *in vitro* can be confirmed *in vivo*, a panel of 20 CCA samples, i.e., 14 ICC and 6 combined CHC samples, as well as 2 non-tumor samples was investigated with regard to the correlation between *SLCO1B3* mRNA expression and the expression of miR-509 and miR-656. Expression data were obtained from the dataset E-GEOD-32958 that contains the mRNA and miRNA profile in cholangiocarcinoma [18]. Using the ArrayExpress Archive of functional genomics data obtained in ICC, significant inverse correlations were observed for OATP1B3 and miR-509-5p, which were not seen for miR-509-3p and miR-656-3p (Fig. 5A to C). This further supports our findings in Huh-7 cells that miR-509-5p exerts an inhibitory effect on OATP1B3 expression.

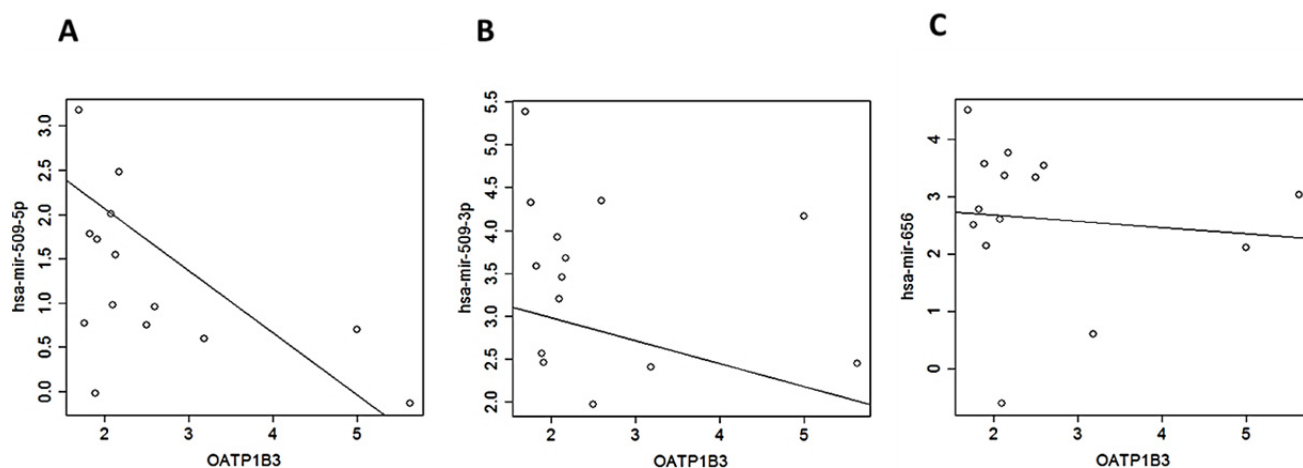


Figure 5: A significant inverse correlation was observed for miR-509-5p and OATP1B3 in Pearson's correlation analyses performed in 14 intrahepatic cholangiocarcinoma samples (E-GEOD-32958, $p=0.048$, coefficient -0.54). These results were confirmed in Spearman's rho analyses ($p=0.046$), when taking a non-normal distribution of OATP1B3, as tested according to Shapiro-Wilk, into consideration.

Discussion

This study investigates to what extent miRNAs modulate the expression of OATP1B3. We show that miR-509 and miR-656-3p significantly suppress OATP1B3 expression at both the mRNA and protein level. Our findings provide a mechanism for the inter-individual variability in OATP1B3 expression that has been repeatedly observed in different disease and tissue types.

The observation that miR-509-5p, miR-509-3p and miR-656-3p suppress OATP1B3 expression at the mRNA and protein levels suggests that these miRNAs both inhibit and degrade *SLCO1B3* mRNA. The *in silico* analysis of the *SLCO1B3*-3' UTR revealed putative binding sites for miR-509-3p and miR-656-3p and luciferase gene reporter assays showed that these two miRNAs appear to bind to the predicted binding sites. The OATP1B3 expression data obtained by RT-PCR and western blot analysis in Huh-7 cells suggest that OATP1B3 is also suppressed by miR-509-5p. This is further supported by correlation analyses performed in a panel of intrahepatic cholangiocarcinoma samples, in which miR-509-5p expression was significantly inversely correlated with the expression of OATP1B3. As this correlation was distinctly observed in ICC and not in mixed samples composed of ICC and HCC, it can be speculated that the miR-509-5p/OATP1B3 interaction plays a considerable role in ICC.

The impact of miR-509-5p on OATP1B3 expression could have consequences for the therapeutic efficacy of anti-cancer drugs. As shown both *in vitro* and *in vivo* in mice, the level of OATP1B3 expression is a determinant of the uptake of platinum drugs used, for example, for the treatment of hepatocellular or cholangiocarcinoma [3]. The effect of miR-509-5p on OATP1B3 expression could, therefore, affect the distribution and accumulation of these drugs in cancer cells. Although *in vitro* effects on OATP1B3 expression comparable with those of miR-509-5p were observed for both miR-509-3p and 656-3p, no inverse correlations between these two miRNAs and OATP1B3 expression were seen in ICC or CHC, suggesting a weaker effect of these miRNAs on OATP1B3 expression in intrahepatic cholangiocarcinoma. Sekyia et al. claimed that ICC is mainly generated by biliary lineage cells derived from hepatocytes, rather than cholangiocytes [19]. ICC and HCC may therefore share the same cellular origin of hepatic stem cells [18]. But, as shown by our data, the epigenetic miRNA-dependent regulatory network seems to differ in both liver cancers. It would be interesting to study the miR-509/miR-656/OATP1B3 interplay in a larger cohort of HCC samples.

MiRNA-509 has repeatedly been reported to be involved in regulatory processes that control cell migration and cell growth in different forms of cancer. Both miR-509-3p and miR-509-5p are thought to be tumor suppressors in renal cell carcinoma and have been shown to suppress the development of brain metastases in breast cancer [20-22]. A suppressive effect of miR-509-5p and miR-509-3p on tumor growth has also been observed in hepatoma and HeLa cell lines [23,24]. Ren et al. revealed that miR-509-5p joins the MDM2/p53 feedback loop in hepatoma cell lines and suggested tumor-suppressive effects of miR-509 [23]. These data confirm an important role for miR-509 in epigenetic regulation, particularly in tumorous tissues.

MicroRNA-656 belongs to a cluster of miRNAs that is decreased in different forms of cancer, including glioblastoma multiforme, renal clear cell carcinoma and invasive breast carcinoma [25]. Consistent with these results, Yin and colleagues observed a downregulation of this miRNA cluster in HCC, which they hypothesized to be mediated by hepatocyte nuclear factor 4 alpha (HNF4a) [26]. In line with the observation that OATP1B3 appears to be highly expressed in extrahepatic tumor entities such as breast cancer, colon cancer, lung or bladder cancer, miR-656 seem to have a stronger impact on extrahepatic, cancerous OATP1B3 expression [27]. In HCC, OATP1B3 expression is decreased in tumorous as compared to non-tumorous samples [28], which, however, still does not exclude a relevant fine-tuning effect of miR-656 on OATP1B3 gene regulation in this type of cancer.

In summary, we show that the miRNAs miR-509 and miR-656 regulate OATP1B3 expression both in a hepatocyte-derived cell line and at the level of gene expression in human intrahepatic cholangiocarcinoma. These findings contribute to our understanding of the high inter-individual differences in OATP1B3 protein expression observed in normal liver and different tumor entities.

Author contributions

J.M., R.K., and G.A.K.-U. participated in research design; R.K., T.C.S., N.D., and J.M. conducted experiments; J.M., R.K., and A.B. performed data analysis; J.M., G.A.K.-U., and H.B.S. wrote or contributed to the writing of the manuscript.

Footnotes

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V. GENERAL DISCUSSION AND OUTLOOK

In this work we elucidate how miRNAs play a crucial role within the signaling pathways modulated by BAs. We demonstrate a bidirectional interaction between FXR, FXR target genes and miRNAs with physiological and pathophysiological consequences for the human digestive tract. These findings support the hypothesis that miRNAs are promising drug targets and agents for the therapy of diseases affecting the liver or intestine. However, as miRNAs are only small molecules within a large and complex epigenetic regulatory network, the risk for miRNA-associated off-target effects should not be neglected.

1. The BA-FXR axis interferes with the endogenous miRNA network

In Chapter 1 we showed that BAs influence the expression pattern of genes and miRNAs important in bile acid homeostasis, lipid and drug metabolism. As CDCA is the most potent endogenous ligand and activator of FXR, in comparison to other endogenously expressed BAs, we chose CDCA in a concentration within physiological range for study performance. Lew et al. showed that within the concentration of 50 μ M CDCA the most potent FXR transactivation is achieved [1]. Additionally, CDCA is an appropriate model substance for drugs in development showing a CDCA-similar structure (e.g. the FXR agonist INT-747). The link between BAs and their FXR-dependent influence on lipid metabolism or cell cycle regulation can be confirmed by our *in vitro* study. As an example, we will focus here on the well described interplay between FXR and its overall suppressive effect on miR-34a expression [2], which we could verify by our data. We observed that miR-34a is *the* p53-regulated miRNA influenced by the BA-FXR axis in hepatocytes. In contrast, miR-192 or miR-509 expressions are not CDCA-dependently changed. This BA-FXR/miR-34a interplay may be an explanation for the anti-apoptotic FXR effects observed in several liver diseases. Looking more into study detail, the miRNA-associated fine-tuning effects on the expression of target genes can be elucidated. The BA detoxifying and drug metabolizing enzyme CYP3A4 has been shown to be indirectly miRNA-dependently regulated over RXR α , as RXR α is directly targeted by miR-34a [3]. Both, miR-34a and CYP3A4 expressions were significantly negatively regulated by the BA-FXR axis in human hepatocytes [4]. The strong inverse correlation between miR-34a and CYP3A4, however, reveals that miRNAs are still able to fine-tune the expression of their target genes, even when the expressions of all interaction partners are treatment- or disease-dependently downregulated.

2. FXR and its target genes are directly and indirectly regulated by miRs

Until now, no epigenetic, miRNA-associated, regulatory mechanism of FXR expression has been shown to have potential implications for altered BA homeostasis and cell apoptosis. Disturbed signaling pathways of cell cycle regulation are described to play a crucial role in several diseases of the human digestive tract such as BA-induced liver injuries, NAFLD or cancer. As miR-192 and FXR are well known to be key players in apoptotic processes, their interaction may be important in the pathomechanism of several hepatic or digestive diseases. To increase the mechanistic value of the miR-192/FXR interplay, transfection experiments shown in Chapter 2 were further confirmed in HepG2 and HT-29 cells (data not shown). As aforementioned, even if the expressions of miR-192 and FXR seem to be simultaneously decreased in colon cancer [5,6], a fine-tuning miR-192-dependent regulatory effect on FXR expression is still possible in this type of cancer. This hypothesis is in line with the results obtained in the correlation analysis between miR-192-3p and FXR expression, where we analyzed 27 adenocarcinoma tissue samples. As the role of FXR in cell growth and cancerogenesis is still controversially discussed, future *in vivo* studies have to elucidate to what extent the miR-192/FXR interplay supports or inhibits tumor pathogenesis. In Chapter 2 we could confirm that miR-192-3p significantly decreases proliferation of Huh-7 and Caco-2 cells. However, only in the hepatoma cell line a partial FXR-dependent effect was observed. Further studies verified that miR-192 FXR-independently impairs cell viability of Huh-7 cells and regulates the expression of the cell cycle regulators cyclin D1 and c-jun (Chapter 2, Suppl. Fig. 3 and 4). These findings are in line with the described pro-apoptotic role of miR-192 [7]. The study of Roy et al. confirmed that miR-192 is involved in the regulation of cell death during oxidative stress-induced liver injury in mice [8]. We therefore conclude that the miR-192-dependent anti-proliferative effects on Huh-7 and Caco-2 cells may have their origin in the cell cycle regulatory role of miR-192 and to a certain extent in the interplay of miR-192 and FXR. These miR-192-associated effects are underlined by *in vivo* data, showing e.g. that miR-192 expression levels are increased in NAFLD, in which cell death counts as a disease key feature [9]. In contrast, expression of miR-192 was significantly suppressed in HCC tissue compared with non-tumorous tissue [10]. We therefore speculate that cancer cells may decrease the endogenous miR-192 expression due to its negative effects on cell viability. Comparable effects and the same expression behavior in NAFLD and HCC were observed for miR-34a, another p53-regulated miRNA [11,12].

In this work we further showed that miR-192 exhibits potentially disturbing effects on BA homeostasis. MiR-192-3p significantly downregulated the expression of FXR target genes

secondary to reduced FXR expression in Huh-7 cells - genes known to play a crucial role in the intracellular, anticholestatic compensatory response of hepatocytes: BSEP, MRP2, MDR3, OST α/β and SHP (Chapter 2, Suppl. Fig. 1). However, intracellular [^3H]-TCA levels did not significantly change upon transfection of Huh-7 cells with miR-192-3p (Chapter 2, Suppl. Fig. 2). The results obtained from the bromsulphthalein-treated cell conditions confirm that TCA is not a specific marker for BSEP-controlled BA efflux in Huh-7 cells. Due to the study of Kullak-Ublick et al. and to our data, we assume that TCA uptake into Huh-7 cells is mainly OATP-mediated [13]. As NTCP is not expressed in Huh-7 cells and OATP1B3 expression was the only hepatic OATP transporter influenced by miR-192, we conclude that Huh-7 cells are not the optimal model cell line to study the miR-192-dependent effect on BA homeostasis. OATP1B3 seems to be the predominant BA transporter being responsible for the TCA equilibrium in this hepatoma cell line. But as the relevance of OATP1B3-dependent BA uptake into hepatocytes is believed to be negligible *in vivo*, an important role of the miR-192/FXR interaction on BA homeostasis has still to be further investigated in future studies.

We showed that miR-192-3p and -5p regulate the expression of FXR and FXR target genes and we revealed pathological implications of this interplay on hepatic and digestive diseases such as intrahepatic cholestasis, NAFLD or cancer – 1) via alteration of BA homeostasis, 2) via disturbed direct effects of FXR or 3) via FXR-independent effects of miR-192 – due to its potential impact on cell cycle regulation, inflammatory processes or lipid metabolism. The proliferation experiments and Suppl. Figures 3-4 in Chapter 2 illustrate that the miR-192-associated effects on cell cycle regulation and cell viability are to some part FXR-dependent – via e.g. disturbance of BA homeostasis – and to another part FXR-independent – via e.g. the impact on JNK/c-jun pathway. As cyclin D1 and the JNK/c-jun pathway are also playing a role in enhanced cell proliferation, the impact of miR-192 on these pathways have to be investigated in better fitting transfection models. From this cell cycle-regulatory impact, undesirable pleiotropic effects of potential miR-192 therapeutics cannot be excluded

Besides hepatocytes, miR-192 and FXR are also expressed in cholangiocytes, whereas FXR expression e.g. in hepatic stellate cells is absent, which makes a direct role of the miRNA/FXR interplay on the hepatic fibrotic response less likely [14,15].

Shifts in microRNA expression can lead to altered drug pharmacokinetics by deregulated expression of drug metabolizing enzymes and transporters. In this work we showed that OATP1B3 expression is directly influenced by miR-509-3p, -5p and miR-656. Additionally, miR-192-3p and -5p suppress OATP1B3 expression in a FXR-dependent manner. We

illustrate that a specific gene is directly and indirectly regulated by five miRNAs in its expression. Considering the enormous amount of >140 *in silico* predicted miRNAs binding to the 3'UTR of OATP1B3 (www.microrna.org), we probably just show here a small section of the whole endogenous, complex miRNA network being involved in the regulation of OATP1B3 expression. As OATP1B3 is a drug transporter important for the cellular uptake of several standard chemotherapeutics (e.g. sorafenib against HCC; platin derivatives or irinotecan against colorectal cancer), shifts in miRNA expression as observed e.g. during cancerogenesis can consequently lead to an altered efficacy of these drugs [16-18]. This fact particularly has to be considered in current drug development, where miRNAs are discussed as potential drug targets against different types of cancer, used in monotherapy or in combination with current standard chemotherapeutics. As shown by the OATP1B3 example, the “multi-to-one” relationship of several miRNAs regulating the expression of a specific gene further elucidates a potential drug resistance mechanism. By changing the endogenous expression pattern of a miRNA with a mimic or inhibitor, the expression levels of the target gene can still remain unchanged due to a subsequent changed expression of other target gene-regulating miRNAs [19]. Coming back to the OATP transporter, the role of OATP1B3 in chemoresistance is controversially discussed. On the one hand, decreased uptake of chemotherapeutics due to suppressed OATP1B3 expression may lead to a diminished sensitivity to cancer therapy. On the other hand, frequently observed OATP1B3 overexpression in e.g. colorectal adenocarcinoma has been associated with an apoptotic resistance to oxaliplatin in a p53-dependent manner. Lee et al. observed that OATP1B3 overexpression decreases the transcriptional activity of p53 [20]. Based on our findings we suggest that the p53-regulated, apoptotic miRNAs miR-509 and miR-192 are missing pieces in a regulatory feedback loop consisting of p53/miR-509/miR-192 and OATP1B3. This feedback loop may confer apoptotic resistance to OATP1B3-transported, but possibly also to non-OATP1B3-transported chemotherapeutics.

3. Are miRNAs future drug targets for liver and digestive diseases?

MicroRNAs were detected around 1990, but only after 2000 they gained more interest, as they were found to be highly conserved throughout mammalian species and to be able to regulate important biological processes within different organisms [19]. As aforementioned, miRNAs are currently discussed to be high-value drug targets and agents. Former defined “undruggable” proteins can potentially be modified by miRNA-dependent gene regulation.

The ability of miRNAs, to simultaneously regulate a large set of target genes, is discussed to be beneficial in multipathway diseases such as cancer, where several oncogenes can be miRNA-dependently suppressed at the same time.

MiRNA mimics and inhibitors are synthesized as exonuclease-resistant, anti-sense agents, which implicates several pharmacological problems [21]. Firstly, these molecules exhibit limited tissue distribution due to low lipophilicity and rapid excretion into urine. The delivery problem is tried to be solved by advanced formulations (nanoparticles, liposome- or antibody-based methods) or chemical modifications [22]. One serious, often discussed problem regarding miRNA therapeutics includes their pleiotropic effects. A single miRNA can regulate up to hundreds of genes, whereas a single gene can be regulated simultaneously or sequentially by multiple miRNAs - facts we have tried to highlight by this work. Several possible mechanisms of miRNA-dependent regulation of protein expression (translational repression vs. mRNA degradation; binding to the 3'UTR of a target gene vs. binding to the 5'UTR or coding region of a target gene) or the additional epigenetic regulation of the miRNAs itself (methylation of their promoter, histone modification) even further increase the complexity of the miRNA-mediated gene regulatory network *in vivo* [19]. Due to these facts it is not surprising that *in vitro* miRNA studies are not satisfactory reproducible and that the number of subsequent clinical trials is limited [23]. MiRNA-associated off-target effects can only be revealed by an intensified study in good animal models and by performing human long-term studies. Price et al. further mention the miRNA-associated problem of tissue specificity, because miRNAs seem to play distinct roles in different tissues and in different cell types within the same organ [19]. In case of the p53-regulated miRNAs miR-192 or miR-34a as possible miRNA therapeutic agents, targeted drug delivery to the diseased tissue would have to be achieved. A drug delivery into healthy tissue should be avoided as both p53-regulated miRNAs have the potential to disturb the BA-FXR axis and/or decrease cell viability. In general, a miRNA inhibitor is probably more specific, as it exerts only activities, where the targeted miRNA is endogenously expressed. A miRNA mimic can distribute throughout the whole body, unless a targeted delivery can be guaranteed. A targeted delivery formulation to the diseased cell type has to include the attachment of folic acid, peptides or antibodies interacting specifically with antigens on the cell surface of e.g. tumors [22,24]. One example for a tissue-specific miRNA is miR-122, which is predominantly expressed in the liver. MiR-122 binds to the 5'UTR of the hepatitis C virus genome and promotes virus replication in the liver [25]. Miravirsen, a locked nucleic acid antagomir against miR-122, is currently tested in clinical phase II for hepatitis C treatment [22]. In contrast, miR-192 and

miR-34a are expressed in various types of tissue, increasing the risk for undesired adverse drug reactions if used as a compound. MRX34, a liposome-formulated miR-34 mimic has become the first miRNA replacement therapy to reach phase I clinical trials as a potential cancer therapeutic agent [26]. As a known cell cycle regulator and a miRNA, which simultaneously regulates several oncogenes, miR-34 induced a complete HCC regression in mouse models [22,23]. First results obtained in clinical phase I trial confirmed partial responses to MRX34 treatment in HCC patients (www.mirnarx.com). MRX34 tumor uptake is claimed to be enhanced by the particular chemical composition of the liposome. The liposome may turn from an anionic into a cationic state in liver tumor microenvironment, which has a lower pH as compared to healthy tissue [26].

As aforementioned, our work illustrates the problem of “unknown” miRNA-associated off-target effects due to complexity of the endogenous human miRNA network. We showed that one miRNA directly regulates FXR expression, whereas disturbances of the BA-FXR axis may lead to significant changes in the expression of >80 miRNAs within hepatocytes. Considering the fact that one miRNA targets up to hundreds of genes, (long-term) drug safety of a potential miRNA therapeutic that influences FXR expression, is difficult to estimate. This concern can further be expanded to the safety of CDCA-analogical drugs, such as the FXR agonist INT-747.

4. Methodological problems and limitations

In Chapter 1, CDCA was used for FXR transactivation. Therewith, FXR-independent BA effects cannot be actively excluded in data interpretation. A next step would include a FXR-miRNA interaction study by using a more specific FXR activator such as INT-747.

To prove the miR-192-dependent regulation of FXR expression, standard methods were used and optimized (Chapter 2). Thereby, transient miRNA transfections only guarantee a miRNA-dependent target gene regulation for 72-96 h. As we observed miRNA-dependent effects on FXR and OATP1B3 protein levels after 72 h in Huh-7 cells, stable miRNA transfections would be more appropriate to study the functional relevance of the miRNA/mRNA interplay (e.g. for transport assays). Furthermore, the 5' strand of the miR-192 precursor only showed non-significant suppressing effects on FXR protein levels in Huh-7 cells, which might be technically biased by the overall higher, endogenous miR-192-5p expression. The “missing” effects of the miR-192-5p/FXR interplay on proliferation of Huh-7 cells for example, have to be interpreted with caution. Therefore, the effects of the miR-192/FXR interplay on the regulation of BA homeostasis were only investigated for the 3' strand. Moreover, some of the

cell transfections with miRNA inhibitors revealed partially reproducibility problems, dependent on the cell line used (Caco-2 cells were partly problematic) and dependent on cell passage number (higher passage number were more problematic). MicroRNA inhibitors are small, chemically modified single-stranded RNA molecules designed to complementary bind to the target miRNA. We could show that also miR-192-5p, the complementary strand of miR-192-3p, influences FXR expression, which may explain the partial variability of the obtained results as the inhibitors in some outlying experiments also downregulated FXR expression. Given the fact that transfection experiments including miRNA mimics were ab initio good reproducible, further experiments were performed with mimics only.

As aforementioned, *in vitro* studies cannot reflect the whole human endogenous miRNA network involved in gene regulation. Furthermore, the effect of signaling pathways involving several different human organs (e.g. immune response, hormone signaling, BA homeostasis) is not optimally mimicked in cell line studies. Therefore, this work just illustrates a selection of signals induced by miRNA/mRNA interplay with implications for the pathophysiology of liver and digestive diseases. The early performance of *in vivo* studies is necessary in miRNA therapeutic research: 1) for a better understanding of the miRNA(s)/mRNA(s) interplay and its influence on signaling pathways throughout the human body, 2) to decrease the linked risk of miRNA-dependent off-target effects and 3) to guarantee tissue-/cell-specific drug delivery.

5. Outlook

We conclude that a bidirectional interaction between FXR and miRNAs is of importance for physiological and pathological effects on the human digestive system. This work illustrates the complexity of the whole human miRNA network by three examples: 1) the significant impact of the BA-FXR axis activation on > 80 miRNAs in hepatocytes, 2) the influence of miR-192 on the expression of FXR and other target genes in a “one-to-multi”-relationship, and 3) the miRNA-dependent regulation of OATP1B3 expression in a “multi-to-one” relationship. Therewith, the limitations of *in vitro* studies as well as the necessity for early performance of *in vivo* studies have been elucidated.

Regarding the miR-192/FXR interplay, more initial *in vitro* research investigating potential effects on the maintenance of BA homeostasis would be essential. Therefore, primary human hepatocytes or HepaRG cells, a hepatic cell line known to express BSEP and NTCP, would be the cell models of choice for further transport experiments. Additionally, stable viral-based miR-192 transfections should be performed, to guarantee long-term miRNA expression within cells. As we saw a miR-192-dependent effect on FXR protein levels not until 72 h, long-term

effects of this interplay on intracellular BA homeostasis and/or cell proliferation after more than 96 h would be interesting to investigate. Furthermore, the apoptotic role of the miR-192/FXR interplay should be examined including appropriate programmed cell death-experiments such as mitochondrial ATP production, transmembrane potentials, cytochrome c release or caspase activation. A further step would be to test the miR-192 mimic effects in a good mouse model, including also FXR knockout mice. But as the miRNA network is not congruent between mice and humans, early performance of human studies is inevitable. As miR-192 is described to be an apoptotic, p53-regulated miRNA, a targeted miR-192 therapeutic would have to be designed, which affects diseased tissue only. As miR-34a and miR-192 show a similar miRNA expression behavior within multiple liver diseases, the effects on FXR expression and disease progression after co-transfection of both miRNAs would be interesting. Possibly, a liver-specific miR-192/miR-34a inhibitor would be a potential treatment for NAFLD, which may strongly re-establish FXR expression and transactivation, may maintain a non-toxic, physiological BA homeostasis and therefore may decrease apoptosis and progression of the disease.

Potential off-target effects always have to be considered in future miRNA therapeutic development. But probably, not all the possible miRNA-dependent mechanisms of protein expression regulation have been detected so far. Buchan et al. described for example, a miRNA-dependent repressing and enhancing role of mRNA translation dependent on cell cycle state and concomitant RNA binding proteins [27]. These two faces of miRNAs open new questions, which join the pool of questions already existing in the complex miRNA universe. Or as Sevignani et al. described the miRNA network: “MicroRNAs are small stars in the genome galaxy with many surprises still in store” [28].

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VI. CURRICULUM VITAE

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Master's degree and federal state exam in Pharmacy
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PROFESSIONAL EXPERIENCE

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Research assistant and pharmacist at the Department of Clinical Pharmacology and Toxicology, University Hospital Zurich, Zurich, Switzerland

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Teaching assistant at practical courses „Xenobiotic metabolism“ for bachelor students in medicine and master students in biomedicine (Department of Clinical Pharmacology and Toxicology, University Hospital Zurich)

AWARDS

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- 06/2015** 10th Retreat of the PhD Program imMed, Solothurn, Switzerland
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- 05/2013** Annual Meeting of Swiss Society of General Internal Medicine (SSGIM), Basel, Switzerland

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“Look up at the stars and not down at your feet. Try to make sense of what you see, and wonder about what makes the universe exist. Be curious.” - **Stephen Hawking**